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New insights into the mechanisms of neutrophil extracellular traps (NET) formation and monosodium urate crystal (MSU)-induced neutrophil responses

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Thèse présenté(e) à la Faculté de médecine et des sciences de la santé
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RÉSUMÉ

Mécanismes sous-tendant la formation des NETs et les réponses des neutrophiles aux cristaux d'urate

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Les neutrophiles représentent un élément essentiel du système immunitaire inné. Ils sont les premiers leucocytes à migrer au site d'inflammation. Une fois au site inflammatoire, les neutrophiles effectuent une multitude de réponses fonctionnelles : la phagocytose, la dégranulation, la flambée oxydative ainsi que la production de médiateurs lipidiques et de cytokines. En plus de ces réponses, les neutrophiles produisent aussi des trappes extracellulaires (NETs). La production de NETs a initialement été décrite comme une réponse antimicrobienne, mais des évidences récentes ont montré son implication dans le développement de plusieurs maladies (goutte, arthrite inflammatoire, psoriasis, etc.) et dans certains cancers.

Malgré l'importance des NETs, les voies de signalisation contrôlant leur formation en réponse à des stimuli physiologiques ne sont que partiellement élucidées. De plus, les méthodes de quantification couramment utilisées pour étudier la formation des NETS comportent plusieurs désavantages. C'est pourquoi les travaux présentés dans cette thèse utilisent de nouveaux polymères fluorescents se liant uniquement à la chromatine extrudée, qui permettent une quantification spécifique et standardisée des NETs. Cette méthode a permis d'établir l'efficacité relative de plusieurs stimuli physiologiques induisant les NETs. Il a également été démontré, chez les neutrophiles activés avec du GM-CSF, du fMLP, ou du TNF, que l'inhibition des voies de signalisation de Syk et de PI3K bloque la NETose en interférant avec les étapes tardives de la formation des NETs. Par ailleurs, l'inhibition des voies de TAK1, de la MAPK p38, ou de MEK empêche aussi la formation des NETs, mais en affectant cette fois des étapes précoces de la NETose. En revanche, l'inhibition de PKC, des kinases de la famille Src ou de JNK n'a pas d'effet sur la formation de NETs; idem pour l'inhibition de la synthèse protéique ou de la transcription. Quant aux mécanismes impliqués dans la formation de NETs, nous avons vérifié le rôle de la NADPH oxidase dans la NETose induite par des stimuli physiologiques. En utilisant notre nouvelle technique de quantification, nous avons confirmé que la formation de NETs induite par le PMA est abolie par l'inhibition de la NADPH oxydase. Par contre, la formation de NETs en réponse à des agonistes physiologiques (fMLP, GM-CSF, TNF) est indépendante de la production de réactifs oxygénés (ROS). De plus nous démontrons pour la première fois que l'inhibition sélective de PAD4 diminue drastiquement la NETose chez les neutrophiles humains et ce, pour tous les stimuli testés.

Une autre section de la thèse porte sur l'interaction entre les cristaux d'urate monosodique (MSU) et les neutrophiles. Le dépôt de MSU dans les articulations déclenche la goutte, une maladie inflammatoire chronique répandue qui est communément associée à une importante infiltration des articulations enflammées par les neutrophiles. Le MSU induit chez les neutrophiles la production de cytokines et de chimiokines, la formation de ROS, ainsi que la relâche de peptides antimicrobiens et d'enzymes protéolytiques. Les neutrophiles activés par le MSU produisent également des NETs. Bien que plusieurs études aient porté sur les mécanismes contrôlant les effets des cristaux de MSU sur les neutrophiles, notre compréhension des voies de signalisation impliquées demeure fragmentaire. Dans la cadre de la thèse, les

changements génomiques et protéomiques causés par le MSU chez les neutrophiles ont été étudiés. De plus, les rôles de multiples voies de signalisation induites par le MSU chez les neutrophiles ont été examinés. Nous montrons pour la première fois que les neutrophiles peuvent sécréter la chimiokine CCL4 en réponse au MSU. Nous démontrons de surcroît que les cristaux de MSU activent tardivement les facteurs de transcription NF-κB, CREB, et C/EBP et que au moins NF-κB est impliqué dans la production de cytokines. Enfin, nous avons établi que le MSU active les MAPK chez les neutrophiles, que leur activation est tributaire de TAK1 et/ou de Syk, et que ces voies de signalisation participent à la production de cytokines et à la NETose.

Les résultats présentés dans cette thèse avancent substantiellement les connaissances actuelles sur les voies de signalisation et les mécanismes moléculaires contrôlant la synthèse et la relâche des cytokines ainsi que la formation de NETs en réponse à des stimuli physiologiques (MSU, GM-CSF, fMLP, TNF) chez le neutrophile humain. Etant donné le rôle crucial joué par les neutrophiles et leurs réponses fonctionnelles dans plusieurs pathologies, ces découvertes pourraient mener à l'identification de cibles moléculaires nouvelles dans une perspective thérapeutique.

Mots clés : Neutrophiles, Trappes extracellulaires, Signalisation, MSU, goutte

Summary

New insights into the mechanisms of neutrophil extracellular traps (NET) formation and monosodium urate crystal (MSU)-induced neutrophil responses

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Neutrophils are an essential component of the innate immune system. They are the first and most abundant leukocytes to migrate to the site of inflammation. Upon arrival, neutrophils perform various functional responses: phagocytosis, respiratory burst, production of lipid mediators and cytokines, neutrophil extracellular trap (NET) formation, etc.

Despite the importance of NETs, signaling pathways that control NET formation are only partialy understood. Likewise, current quantification methods that are used to study mechanisms of NET formation suffer from significant drawbacks. As part of this thesis, we used new fluorescent polymers that only bind extruded chromatin, allowing a specific and standardized quantification of NETosis that could not be easily performed before. This approach allowed us to rank the relative potency of various physiologic NET inducers reliably, and to show that inhibition of the Syk or PI3K pathways blocks NETosis by acting upon late events in NET formation. By comparison, inhibition of the TAK1, p38 MAPK, or MEK pathways also disrupted NETosis, but by acting on early events. In contrast, inhibiting PKC, Src family kinases, or JNK failed to prevent NET formation; inhibitors of translation and transcription (cycloheximide and actinomycin D respectively) were likewise ineffective. The role of the NADPH oxidase in NET formation was also revisited. We confirmed that pharmacological PKC activator (PMA) induced NET formation that depends on the oxidase, contrarily NETosis occurring in response to physiological agonists is ROS-independent. Finally, we demonstrated for the first time that selective inhibition of protein arginine deiminase 4 (PAD4) potently prevents NETosis in human neutrophils by all stimuli tested.

The second part of my thesis is devoted to revisiting how monosodium urate (MSU) crystals interact with neutrophils. Deposition of insoluble MSU in joints is known to trigger gout, a prevalent chronic inflammatory disease that is commonly associated with massive neutrophil infiltration. Several studies have focused on the mechanisms underlying the numerous actions of MSU crystals towards neutrophils. However, our knowledge of the signaling pathways involved remains fragmentary. During the work on this thesis, genomic and proteomic changes triggered by MSU in neutrophils were examined. Additionally, the role of various signaling pathways in MSU-induced neutrophil functional responses was studied. This allowed us to show for the first time that neutrophils can secrete CCL4 in response to MSU. Accordingly, it was demonstrated that MSU crystals belatedly activate transcription factors NF-κB, CREB, and C/EBP, and at least the former is involved in cytokine generation. Additionally, we showed that MSU activates MAPKs in neutrophils, that they are under the control of TAK1 and/or Syk, and that they participate in cytokine generation and NET formation.

Data obtained during the work on this thesis substantially extends current knowledge of neutrophil signaling pathways controlling the cytokine generation and NET formation. Taking in to account the involvement of neutrophil functional responses in various pathologies (and especially gouty arthritis), our findings identify potential molecular targets that could be exploited for therapeutic intervention.

Keywords: neutrophils, extracellular traps, signaling, MSU, gout

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LIST OF ABBREVIATIONS

3-MA	3-methyladenine
5-LO	5-lipoxygenase
FLAP	5-lipoxygenase activating protein
5-HPETE	5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid
ATP	adenosine triphosphate
ANCAs	anti-neutrophil cytoplasmic autoantibodies
ApoB	apolipoprotein B
ApoE	apolipoprotein E
AA	arachidonic acid
AS	Atherosclerosis
ACPAs	autoantibodies to citrullinated protein antigens
AZU	azurocidin
CG	cathepsin G
CGD	chronic granulomatous disease
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DCs	dendritic cells
DNase	deoxyribonuclease
DPI	diphenyliodonium
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GPCRs	G-protein coupled receptors
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte–macrophage colony-stimulating factor
GPA	granulomatosis with polyangiitis
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
ITAM	immune tyrosine activation motif
LTF	lactoferrin
LT	leukotriene
LDG	low-density granulocyte
LFA-1	lymphocyte function-associated antigen 1
LYZ	lysozyme
MPA	microscopic polyangiitis

MSU	monosodium urate
MPO	myeloperoxidase
NK	natural killer
NE	neutrophil elastase
NEi	neutrophil elastase inhibitor
NETs	neutrophil extracellular traps
NADPH	nicotinamide adenine dinucleotide phosphate oxidase complex
NO	nitric oxide
NRTK	non-receptor tyrosine kinase
NSAIDs	non-steroidal anti-inflammatory drugs
PRR	pathogen recognition receptor
PADs	peptidyl arginine deiminases
PMA	phorbol 12-myristate 13-acetate
PI3K	phosphatidylinositol 3-kinase
PS	phosphatidylserine
pDC	plasmacytoid dendritic cells
PAF	platelet-activating factor
PUFA	polyunsaturated fatty acid
PG	prostaglandin
PR3	proteinase 3
ROS	reactive oxygen species
RSV	respiratory syncytial virus
RA	rheumatoid arthritis
SLPI	secretory leukocyte proteinase inhibitor
SFL	synovial fibroblast
SF	synovial fluid
SLE	systemic lupus erythematosus
TX	thromboxane
TEM	transendothelial migration
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

INTRODUCTION

1. Innate immunity and neutrophils

Innate immunity is a complex aspect of host defense comprised of many cell types and a myriad signaling molecules, working together against injury or infection. It is an old evolutionary construction that has proved remarkably effective against most types of micro-organisms. Innate immunity tackles most common viruses, bacteria, fungi, and protozoa, as well as host cells undergoing damage or malignant transformation, all the while tolerating healthy host cells and normal flora (Hopcraft and Damania, 2017; Machado *et al.*, 2004). Components of innate immunity are the first to intervene upon pathogen invasion, thereby preventing infection and restoring homeostasis. When incapable of eliminating the cause of infection, innate immune cells attempt to restrain pathogen spreading, while triggering the adaptive immunity machinery. Adaptive immunity is by comparison antigen-specific and has memory. It is therefore more efficient than the robust innate immunity, but takes much longer to be mobilized. As mammals are continuously faced with a varied microbial challenge, proper functioning of innate immunity is critical for maintaining health. Inappropriate functioning of innate immunity leads to a broad spectrum of disorders, ranging from mild, tolerable chronic conditions to life-threatening diseases (Kumar and Sharma, 2010; Machado *et al.*, 2004; Mantovani *et al.*, 2011).

Although quite sophisticated, the innate immune response boils down to crosstalk and interactions between its main components: anatomical barriers, phagocytic leukocytes, the

complement system, and mediators such as cytokines (Alberts *et al.*, 2002). That said, polymorphonuclear leukocytes, or neutrophils, are often considered the main effectors of innate immunity, as they are the most numerous population of leukocytes patrolling the bloodstream and usually the first to infiltrate the tissues (Kolaczkowska and Kubes, 2013).

Neutrophils are exquisitely adapted to encounter pathogens primarily due to high mobility and deformability. This is in part facilitated by a flexible multi-lobe shaped nucleus that ensures the cell's challenges of moving through cell junctions or the extracellular matrix (Carvalho *et al.*, 2015). Originating from the bone marrow, neutrophils are released in the bloodstream, from where they migrate to the site of injury. Neutrophils are approximately 12-14 μm in diameter, produced each day in huge quantities (10^{11}) in response to growth factors such as granulocyte colony-stimulating factor (G-CSF) (Dancey *et al.*, 1976; Hong, 2017). The extensive production of these cells is offset by their short lifespan that ranges from 8 to 12 hours (Summers *et al.*, 2010). In the absence of infection and inflammation, neutrophils die by spontaneous apoptosis (Geering and Simon, 2011). The mechanisms that regulates production and neutrophil quantities remain largely unknown (Vietinghoff and Ley, 2008).

Neutrophils deploy three main strategies to combat infection. First response that involves clearing extracellular pathogens by neutrophils is phagocytosis, a process of ingestion of microorganisms and fusing them with cytotoxic vacuoles (Lee *et al*., 2003a). Second, if the pathogens are too big or there are too many of them, neutrophils start to produce large amounts of lytic enzymes and reactive oxygen species (ROS) into extracellular space (degranulation) (Chen and Junger, 2012; Lacy, 2006). Third step, forming neutrophil extracellular traps (NETs). NETs constrain pathogens and allow for the targeted release of granule contents

(Brinkmann *et al*., 2004a). Following microbial degradation, neutrophils usually undergo apoptosis and elimination by macrophages (Haslett *et al.*, 1995; Silva, 2011).

Due to their high cytotoxic potential, neutrophils must be strictly controlled by multiple mechanisms to prevent harm to the tissue (Kruger *et al*., 2015). Circulating neutrophils exist in an incompletely activated state, and are only activated in the vicinity of the infection (Condliffe *et al.*, 1998). Stimuli include many host and pathogen-derived molecules, including cytokines, chemokines, growth factors and leukotrienes (Vogt *et al.*, 2018). Neutrophils express an extensive repertoire of surface receptors recognizing the latter, whose binding triggers functional responses (Futosi *et al.*, 2013).

Neutrophil activation enhances the production of cytotoxic agents and mobilization of secretory vesicles, as well as adherence and chemotactic potential. This activation process is reversible, enabling the neutrophil to return to a semi-activated state and migrate back to vasculature in case of false alarm (Condliffe *et al.*, 1998; Kitchen *et al.*, 1996). The complete molecular mechanisms behind neutrophil migration and activation are still mostly unknown, but evidence suggests that these are multistep processes and that several pathways are involved. What seems apparent is that neutrophils are highly phenotypically plastic. According to specific microenvironment demands, they can modulate their surface molecules and take on specialized tissue-specific roles (Amulic *et al.*, 2012; Kumar and Sharma, 2010; Mantovani *et al.*, 2011; Nathan, 2006; Sadik *et al.*, 2011).

2. Neutrophils in inflammation

Being major players of innate immunity, neutrophils actively participate in the inflammatory response that involves a highly conserved cascade of events, and mobilizes an assortment of leukocytes and signaling molecules (Rosales, 2018). Various factors can trigger the inflammation cascade, most notably infection, injury or exposure to foreign particles. Inflammatory process is commonly seen as a defense mechanism (protection against pathogens), however it can also harm the host, causing collateral tissue damages, and septick shock (Medzhitov, 2008).

Acute inflammatory reaction starts with vascular response which is characterized by alteration of the microvasculature near the damaged site, under the action of inflammatory mediators (Pober and Sessa, 2015). Acute inflammatory mediators: histamine (mast cells and basophils) and serotonin (platelets) induce changes in vascular flow followed by vasodilatation and oedema (Rankin, 2004). Vasodilation (widening of the blood vessel), is mediated by nitric oxide (NO) and prostaglandins (PGI_2, PGD_2, PGE_2 and $PGF_{2\alpha}$) produced by epithelial cells located on the intima of blood vessels. Oedema (fluid entry into the tissue) is a result of actions of histamine, bradykinin, leukotrienes, complement components, substance P and platelet-activating factor (PAF). Together, these factors, mediate delivery of soluble mediators and inflammatory cells to the site of injury (Friedl *et al.*, 1989; Granger and Senchenkova, 2010; Sandoo *et al.*, 2010; Sherwood and Toliver-Kinsky, 2004). Additionally, many different residential and recruited cell types at the inflammatory site can produce chemotactic cytokines (LTB4, CXCL8, CCL5, CXCL3, CXCL5, CXCL6 etc.) which induces the leukocyte influx (Krishnaswamy *et al.*, 1999; Sokol and Luster, 2015).

Neutrophils are the first and the most abundant leukocytes to swiftly migrate from circulation to the infected area (de Oliveira *et al.*, 2016). Chemoattractants (CXCL2, CXCL8, MCP1, LTB4) released by macrophages residing in the tissues, epithelial cells and possibly other cell types, depending from the origin of affected tissue, alert circulating neutrophils causing a rapid influx to the site of infection (Beck-Schimmer *et al.*, 2005; Filippo *et al.*, 2008). The cellular events accompanying the migration process to the inflamed tissue operate in a cascade and can be divided into several steps: margination, rolling, adhesion, diapedesis (transendothelial migration) and chemotaxis. Margination is a neutrophil movement from central blood stream towards periphery of the vessel mediated by fluid stasis at the site of inflammation. Margination promotes weak adhesive interaction between neutrophils and vascular endothelium (Sherwood and Toliver-Kinsky, 2004). Then neutrophils start to roll along the endothelial surface of postcapillary venules in search for an appropriate place to extravasate to the interstitium in the vicinity of the damaged tissue (Kim and Luster, 2015a). The motion of neutrophils along the chemokine gradient in this state is often described as "rolling" mediated by the interaction between selectins (expressed on endothelial cells) and their corresponding ligands (on leukocytes) (Alon *et al.*, 1996; Rosen, 1993). During rolling, neutrophils continue to collect inflammatory cues. Signals from inflammatory chemokines transmitted to neutrophils induce G-protein coupled receptor- (GPCRs)-mediated integrins activation (Herter and Zarbock, 2013). Integrins like, Mac-1, ICAM-1 and VCAM-1 become activated, augmenting neutrophil affinity towards endothelial cells. These interactions, induce cytoskeletal rearrangement and polarization of the neutrophils, which lead to reduction of neutrophils rolling velocity and eventually arrest on endothelium (Voisin and Nourshargh,

2013; Williams *et al.*, 2011). The changeover from migratory state to adhesion is highly dependent on the time the neutrophil spent in contact with activated endothelium. The stabilization of this connection activates signaling cascades that initiate transendothelial migration (TEM). Neutrophils can transmigrate between endothelial cell junctions (paracellular TEM) or through the body of the endothelial cells (transcellular TEM). Likewise, studies have demonstrated that the majority of TEM events happen via paracellular route (~70-90%) (Nourshargh and Alon, 2014). The complete mechanism of TEM remains elusive, however several endothelial transmembrane proteins (PECAM-1, ICAM-1, VE-cadherin, JAM and CD99) have been shown to play a role (Alcaide *et al.*, 2009; Ley *et al.*, 2007; Williams *et al.*, 2011; Zen and Parkos, 2005). After TEM, following strong chemotactic gradient, neutrophils arrive at the inflammation focus, where their full activation coordinates a robust response (Liew and Kubes, 2019).

Under normal conditions, neutrophils are short-lived cells, a property fit for avoiding unneeded inflammation. However, in an inflammatory state, neutrophils encounter a mixture of pro-inflammatory mediators (such as granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony – stimulating factor (G-CSF), TNF-α, bacterial components, C-reactive protein, serum amyloid A) that activates them and prolong their lifespan (delayed apoptosis) (El Kebir and Filep, 2010; Greenlee-Wacker, 2016; Takano *et al.*, 2009). In the form of positive feedback, activated neutrophils attract even more neutrophils and other immune cell types to the proximity. This process is modulated by variety of pro-inflammatory cytokines and chemokines secreted by neutrophils (Selders *et al.*, 2017; Tamassia *et al.*, 2018). Neutrophil functional responses (phagocytosis; radical oxygen generation; degranulation;

cytokine, chemokine, lipid mediators production; and NETs) induced at the site of inflammation will be discussed in more detail further.

Beyond their prominent role in the acute phase of inflammation, neutrophils also play important role in the resolution phase (El Kebir and Filep, 2010b). The induction of apoptosis in spent neutrophils and their subsequent phagocytosis by macrophages (efferocytosis) is central to the inflammation resolution. Aging neutrophils downregulate a number of membrane proteins including CD16 (FcγRIII), CD31 (PECAM-1), CD32, CD35 (Complement receptor 1), CD43, CD45, CD44, CD47 (IAP), CD50 (ICAM-3), CD55 (Complement regulatory protein DAF), CD62L (L-selectin), CD63, CD66b, CD87 (uPAR), CD88 (C5a receptor), and CD120a (tumor necrosis factor receptor 1), thus significantly changing the apoptotic neutrophil surface (Greenlee-Wacker, 2016a). Additionally, dying neutrophils secrete mediators (annexin A1, lactoferrin, lipoxins, resolvins) that inhibit further neutrophil recruitment and promote neutrophil apoptosis and clearance by macrophages (Greenlee-Wacker, 2016b). Upon efferocytosis of apoptotic neutrophils, macrophages downregulate production of proinflammatory cytokines and lipid mediators (IL-8, GM-CSF, LTB4, IL-1 β, TNF) and switch to an anti-inflammatory program. Anti-inflammatory macrophages start to produce TGF-β and prostaglandin E2 (PGE2). This represents a turning point that triggers a deep change in the macrophage secretion profile and promotes resolution (Fadok *et al*., 1998; Ortega-Gómez *et al.*, 2013; Serhan *et al.*, 2008; Sugimoto *et al.*, 2016).

Although inflammation has been heavily researched at the cellular and molecular levels, our understanding of the process continues to evolve. It is important to underline that each

tissue has a specific signature of the inflammation process, as a result of a unique microenvironment interacting locally with a highly plastic and adaptable immune system. This can also be clinically relevant, as specific manifestations of the disease may require appropriate medical treatments.

3. Neutrophil functional responses

3.1. Phagocytosis

Phagocytosis is a process by which neutrophils and other immune system cells internalize solid particles larger than 0.5 μm into intracellular vacuoles called phagosomes. Although the process was discovered more than 100 years ago, our understanding of the complex molecular mechanisms underlying phagocytosis is incomplete (Rosales and Uribe-Querol, 2017).

Neutrophils can engulf both opsonized and non-opsonized particles, involving different receptors and pathways (Hiemstra and Daha, 1998). Nonopsonized receptors, also known as pathogen recognition receptors (PRRs), can directly recognize molecular groups on the surface of phagocytic target. Thus, there are several families of PRRs expressed by neutrophils: Toll-like receptors (TLR1,2,4,5,6,8,9); C-type lectins (Dectin-1, Mincle, MDL-1 Mcl, CLEC-2); Nod-like receptors (NOD2, NLRP3); and RIG-like receptors (RIG-1, MDA5) (Kumar *et al.*, 2011). However, ingestion of pathogenic particles by neutrophils is more effective if recognized by opsonins that adhere to foreign particles, promoting their recognition and subsequent phagocytosis (Verhoef, 1998). Neutrophil phagocytosis of the opsonized particles is mainly mediated by two classes of receptors. Receptors that recognize

the Fc portion of immunoglobulin G (IgG) and those that recognize fragments of the complement. Neutrophils express different complement receptors: integrin family [CR3 (CD11b/CD18) and CR4 (CD11c/CD18)]; GPCR (C5aR); mannose-dinding lectin and surfactant protein A (C1qR); SCR family receptor (CR1) (DeFranco *et al*., 2007; Sengeløv, 1995). Although they involved in neutrophil phagocytosis their signaling pathways remain uncertain (Futosi *et al.*, 2013; Petty and Todd, 1993; Vik and Fearon, 1985, O'shea *et al*., 2008). In contrast, Fc receptors that bind Ig-opsonized pathogens, are the best characterized phagocytic receptors. Neutrophils express various Fc receptors [FcγRIIIb (low affinity IgG receptor, exclusive for neutrophils), FcγRIIa (low affinity IgG receptor), FcγRI (IgG receptor), FcαRI (IgA receptor), FcεRI (IgE receptor), FcεRII (IgE receptor)]. Fcγ receptor family is the most significant for triggering phagocytosis (Fanger *et al*., 1998) (Futosi *et al.*, 2013; Kumar *et al.*, 2011; Lee *et al.*, 2003; Nordenfelt and Tapper, 2011).

Recognition of foreign particles by membrane-bound receptors activates downstream signaling, resulting in polymerization of actin fibers, membrane rearrangement and phagosome formation (Futosi *et al*., 2013b; García-García and Rosales, 2013). At this point, the phagosome is still immature as it has not yet acquired antimicrobial properties. Following internalization, the receptor is recycled back to the membrane, while the entrapped particle resides in the early phagosome. The phagosome then fuses with neutrophil granules, forming a phagolysosome, rich with bactericidal molecules and lytic enzymes (Nordenfelt and Tapper, 2011b). Neutrophil granules can be classified based on their contents to primary containing myeloperoxidase (MPO) and CD63; secondary carrying lactoferrin and CD66b; tertiary that lost CD66b but acquiring gelatinases; and finally, secretory particles encompassing albumin,

Azurophil Granules	Membrane:	CD63; CD68; V-type H^+-ATPase
	Matrix:	Acid β-glycerophosphatase; Acid mucopolysaccharide; α_1-Antitrypsin; α-Mannosidase; Azurocidin/CAP37/heparin binding protein; Bactericidal permeability increasing protein; β-Glycerophosphatase; β-Glucuronidase; Cathepsins; Defensins; Elastase; Lysozyme; Myeloperoxidase; N-Acetyl-β-glycosaminidase; Proteinase-3; Sialidase; Ubiquitin-protein
Specific Granules	Membrane:	CD11b; CD15 antigens; CD66; CD67; Cytochrome b_{558}; fMLP-R; Fibronectin-R; G-protein$_\alpha$-subunit; Laminin-R; NB 1 antigen; 19-kD protein; 155-kD protein; Rap1, Rap2; SCAMP; Thrombospondin-R; TNF-R; Urokinase-type plasminogen activator-R; VAMP-2; Vitronectin-R
	Matrix:	B_2-Microglobulin; Collagenase; Gelatinase; hCAP-18; Histaminase; Heparanase; Lactoferrin[301]; Lysozyme; NGAL; Urokinase-type plasminogen activator; Sialidase; SGP28; Vitamin B_{12}-binding protein;
Gelatinase Granules	Membrane:	CD11b; Cytochrome b_{558}; Diacylglycerol-deacylating enzyme; fMLP-R; SCAMP; VAMP-2; V-type H^+-ATPase; Urokinase-type plasminogen activator-R
	Matrix:	Acetyltransferase; β_2-Microglobulin; Gelatinase; Lysozyme
Secretory Granules	Membrane:	Alkaline phosphate; CR1; Cytochrome b_{558}; CD11b; CD14; CD16 [*] fMLP-R; SCAMP; V-type H^+-ATPase; Urokinase-type plasminogen activator-R; VAMP-2; CD10 CD13 CD45 [*]; C1q-receptor [*]; DAF [*]
	Matrix:	Plasma proteins (including tetranectin)

* This localization is based on kinetics of upregulation in response to stimulation with inflammatory mediators, but has not yet been demonstrated by subcellular localization by immunocytochemistry.

Table 1. Content of Human Neutrophil Granules and Secretory Vesicles. (adapted from (Borregaard and Cowland, 1997, authorization requested order #4661401049529)

alkaline phosphatase, and CD35. The full list of various granules content is summarized in the Table 1. (Borregaard and Cowland, 1997). In addition, formation of neutrophils phagolysosome initiates activation of NADPH oxidase on the phagolysosome membrane, that induces an oxidative burst inside of the phagolysosome. This creates a highly toxic microenvironment for the destruction of microorganisms (Freeman and Grinstein, 2014; Lee *et al.*, 2003; Nordenfelt and Tapper, 2011; Scott *et al.*, 2003). Detailed mechanism of NADPH oxidase activation and ROS production is described in the next section.

3.2. Oxidative burst and degranulation

During phagocytosis, neutrophils significantly increase their oxygen consumption, ultimately resulting in the production of ROS, catalyzed by the nicotinamide adenine dinucleotide phosphate oxidase (NADPH) complex (Figure 1). In resting neutrophils catalytic core of the NADPH complex ($gp91^{phox}$ and $gp22^{phox}$) resides at the membranes of phagosome, secretory vesicles, specific granules and the plasma membrane. Regulatory subunits ($p67^{phox}$, $p40^{phox}$, $p47^{phox}$) reside in the cytosol. During neutrophil activation the cytosolic complex translocates to the membrane and interacts with the catalytic core which results in NADPH oxidase activation. Additionally, GTPase protein, Rac2 binding to catalytic core of NADPH is required for ROS production (Babior *et al.*, 2002; Nguyen *et al*., 2017).

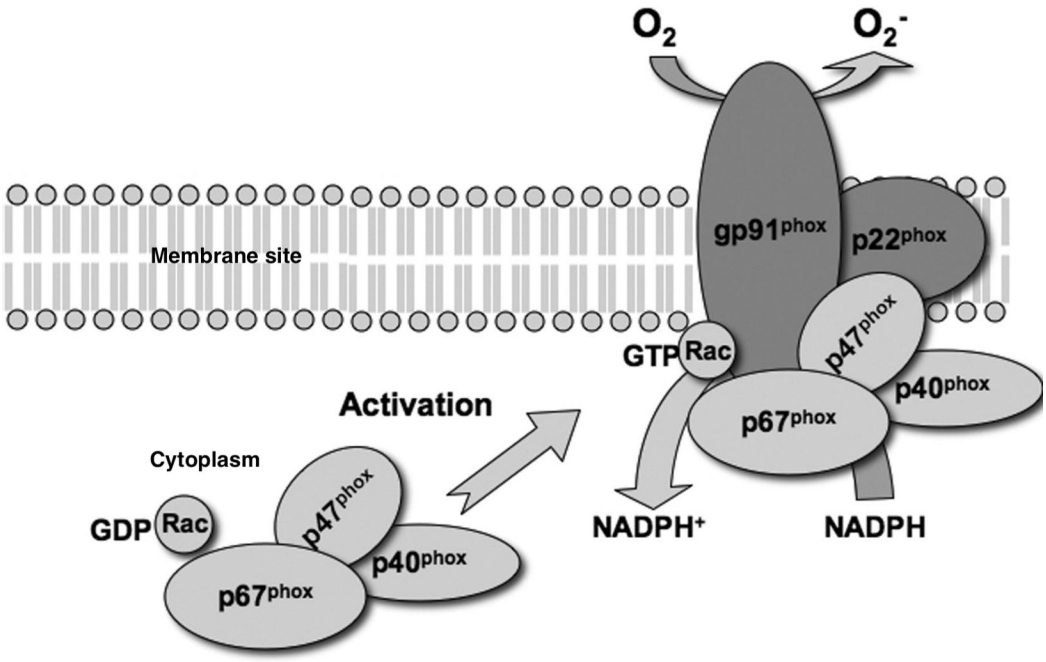

Figure 1. Structure and activation of Nox2 NADPH oxidase.

Activation involves translocation of the cytosolic subunits p47*phox*, p67*phox*, p40*phox* and Rac to the membrane where they bind to cytochrome b_{558}, composed of p22*phox* and gp91*phox* (Nox2) subunits (adapted from Dworakowski et al., 2006, distributed under the Creative Commons Attribution License)

Fully active NADPH oxidase mediates production of ROS: superoxide anions (O_2^-) or hydrogen peroxide (H_2O_2). The ROS may be further reduced by a number of different cellular protection systems, including superoxide dismutase (catalyzes the reduction of O_2^- to H_2O_2), catalase (catalyzes the reduction of H_2O_2 to H_2O), and glutathione peroxidase (catalyzes the oxidation of glutathione by H_2O_2). Likewise, O_2^- can react with nitric oxide (NO) to process very reactive peroxynitrite molecule. This reaction is catalyzed by cellular NO synthase. Hydrogen peroxide can also be metabolized to hypochlorous acid (HOC1) by myeloperoxidase, localized in azurophil granules (Babior *et al.*, 2002; Nguyen *et al.*, 2017; PACHER *et al.*, 2007; Ray *et al.*, 2012).

The process of NADPH assembly that occurs on the cellular membrane inducing ROS production towards extracellular space is called an oxidative burst (also referred as respiratory burst). The oxidative burst is one of the main functional responses that enable neutrophils to protect the host. Apart from their cytotoxic potential, ROS also have a regulatory role, functioning as a molecular switch in cellular redox pathways (Ray *et al.*, 2012). Mutations in genes coding for NADPH oxidase subunits cause severe life threatening chronic granulomatous disease (CGD), underpinning the importance of this enzyme. This implies that the activation of NADPH oxidase has to be strictly regulated as not to cause ROS production in homeostatic state (Chen and Junger, 2012; Groemping and Rittinger, 2005; Nguyen *et al.*, 2017; Roos, 2016).

Neutrophils not only produce ROS into extracellular space, they can also release lytic enzymes, antimicrobial peptides and inflammatory mediators that are contained in the intracytoplasmic granules (Table 1). This process is called degranulation represents another defense mechanism of pro-inflammatory neutrophils. Degranulation can be induced by various mediators (CXCL8, fMLP, C5a, LTB4) and controlled by distinct signaling pathways that include the Src family of tyrosine kinases, β-arrestins, the tyrosine phosphatase MEG2, the kinase MARCK, Rabs and SNAREs, and the Rho GTPase, Rac2. Likewise, it was shown that an increase in intracellular Ca^{2+} and hydrolysis of guanosine triphosphate (GTP) as well as of adenosine triphosphate (ATP) is needed for the translocation and release of granular content. However, the mechanism that control neutrophil degranulation is not well understood (Lacy, 2006; Lacy and Eitzen, 2008; Sheshachalam *et al.*, 2014).

In summary, both degranulation and respiratory burst play an important role in host defence against invading microorganism. However, dysregulation of these functional responses can be implicated in pathologies (sever asphyxic episodes of asthma, rheumatoid arthritis, septic shock, CGD, *etc*.)(Brieger *et al.*, 2012; Lacy, 2006; Sheshachalam *et al.*, 2014).

3.3. Inflammatory mediators

Besides the traditional functional responses discussed in the above paragraphs, neutrophils also can secrete a variety of mediators that allow them to communicate with surrounding microenvironment. Neutrophils are often the first to arrive at the site of inflammation. Thus, in addition to their function for the rapid elimination of the pathogens, neutrophils ~~can~~ also orchestrate the continuation of the immune response and induce migration of other leukocytes to inflamed sites through the production of lipid and polypeptide mediators (Bennett and Gilroy, 2016a; Billingham, 1987; Mayadas *et al*., 2014a; Tamassia *et al*., 2018b).

3.3.1. Lipid mediators

Lipid mediators are compounds formed from polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Bennett and Gilroy, 2016b). Some of the lipid mediators derived from the metabolism of AA, are called eicosanoids (Khanapure *et al.*, 2007). The main eicosanoids generated by neutrophils are leukotriene (LT) B4, PGE2 and thromboxane (T_X) A_2 (Ford-Hutchinson *et al*., 1980; Goldstein *et al*., 1978; Zurier, 1976).

During neutrophil activation an increase in intracellular calcium, provides the translocation of type IV cytosolic phospholipase A_2 ($cPLA_2$) to the nuclear membrane (Lin *et al*., 1992). Then $cPLA_2$ cleaves AA in the ***sn***-2 position of phospholipids. Released AA can be used by several metabolic pathways: the leukotriene formation pathway and the prostanoid formation pathway (Figure 2) (Sala *et al*., 2010).

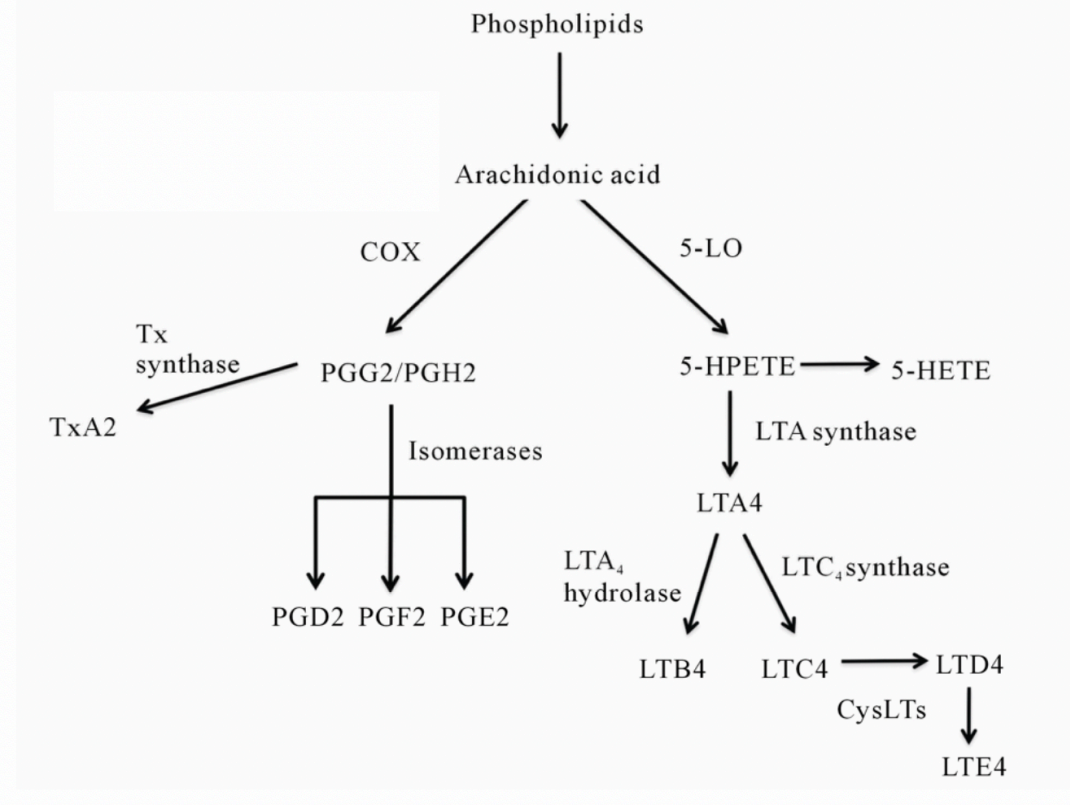

Figure 2. Lipid Mediators.

Prostaglandins (PGD2, PGE2 and PGF2) and Thromboxane A2 (TxA2) are derived from arachidonic acid by cyclooxygenase (COX) mediated pathway. Leukotrienes (LTB4 and Cysteinyl-leukotrienes-LTC4, LTD4 and LTE4) are produced by the action of 5-lipoxigenase (5-LO). (adapted from Bajaj and Ishmael, 2013, distributed under the Creative Commons Attribution License)

Leukotriene formation pathway initiates with the translocation of 5-LO to the nuclear membrane (Ford-Hutchinson *et al*., 1980; Rådmark and Samuelsson, 2009). Produced at the

earlier step AA binds to 5-lipoxygenase activating protein (FLAP) for presentation to 5-LO. Thus, 5-LO catalyzes oxygenation of AA to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE), and further dehydration to the leukotriene A4 (LTA4) (Rådmark and Samuelsson, 2009). Subsequently, LTA4 can be converted to LTB4 by LTA4 hydrolase (Haeggström, 2000). It is known that LTB4 have several effects at the inflammatory site, such as increased vascular permeability and adhesion to endothelial cells, its main action on neutrophils: potent chemoattractant and activator of degranulation (Bennett and Gilroy, 2016; Gimbrone *et al.*, 1984; Khanapure *et al.*, 2007). The 10^{-9} M concentration of LTB4 is sufficient to trigger neutrophil chemotaxis, thus allowing them to move towards a concentration gradient at the site of inflammation (Dos Santos and Davidson, 1993).

Prostanoid (PGs, prostacyclins, T_Xs) formation in neutrophils is mediated by the COX (also known as prostaglandin G/H synthase) enzyme that catalyzes two reactions by which AA is transformed to PGH_2, the common precursor of all prostanoids (St-Onge *et al.*, 2007). There are two known COX isoforms in humans: COX-1 and COX-2. However, COX2-mediated prostanoid formation seems to be more important in the context of inflammation (Dubois *et al.*, 1998). After PGH_2 is formed, it can be isomerized to PGE_2. This reaction can happen in non-enzymatic manner or provided by one or more of several PGE2 synthases. Likewise, thromboxane synthase uses PGH_2 to form T_XA_2. Once produced, these mediators leave the cell spontaneously or by transporters, and activate their respective receptors. Eight G protein-coupled prostanoid receptors have been characterized in humans (Narumiya and FitzGerald, 2001). Both PGE2 and T_XA_2 are produced by neutrophils and have their role during inflammation. Eicosanoid- T_XA_2 has generally pro-inflammatory effects, such as platelet

activation and aggregation, as well as increased expression of adhesion molecules (ICAM-1, VCAM-1, ELAM-1) by endothelial cells (Nakahata, 2008). No reported effects of T_XA_2 on neutrophils were found.

On the other hand, PGE2 can play both pro- or anti-inflammatory role, depending on the situation, the cell and the receptor involved. It can cause vasodilation, participate in edema and fever. In addition, it has been implicated in cytokine production by dendritic cells (DCs) (Yao *et al.*, 2009), thus contributing greatly to the course of inflammation (Legler *et al.*, 2010). An anti-inflammatory role of PGE2 includes suppression of CCL19 production by DCs and monocytes, direct inhibition of IL-2 synthesis and IL-2 receptor expression by T cells, and suppression the cytotoxic activity of natural killer cells (NK) (Nakanishi and Rosenberg, 2013). In addition, PGE2 can inhibit neutrophil inflammatory responses: chemotaxis and superoxide anion production (Armstrong, 1995; Talpain *et al*., 1995). Likewise, PGE2 promotes neutrophil reverse migration (Loynes *et al.*, 2018).

In summary, lipid mediators produced by neutrophils play important pro- and anti-inflammatory role during inflammation (Bennett and Gilroy, 2016b; Stables and Gilroy, 2011). Dysregulated production of lipid mediators is implicated in chronic inflammatory disorders (Chiurchiù *et al*., 2018; Heller *et al*., 1998).

3.3.2. Peptidic mediators (cytokines, chemokines)

Cytokines are crucial signaling molecules mediating information between cellular components of innate and adaptive immunity (Zhang and An, 2007). These are small

molecules produced by immune and non-immune cells, involved in autocrine and paracrine signaling. They have multiple roles, foremost among which is the management of essential homeostatic processes such as inflammation, tissue reparation or angiogenesis (Lackie, 2010). Neutrophils have long been thought to have little or no transcriptional activity or protein synthesis ability; however, data generated in the early 1990s has established that they are potent producers of a diverse palette of biologically active molecules. Neutrophils are both targets and producers of a wide repertoire of cytokines (Cassatella *et al*., 1997; Tamassia *et al*., 2018). They can therefore be involved in physiologically beneficial or detrimental processes. It has been shown that cytokines influence neutrophil behavior in multiple ways, including cytokine production itself, adhesion, phagocytosis, ROS production, degranulation and (more recently) NET formation (Garley *et al*., 2017; Tecchio *et al*., 2014).

The production of cytokines by neutrophils can sometimes be constitutive (as in the case of IL-1ra, IL-18BP, or IL-8) but it is generally triggered upon cell stimulation. Various signals from the local microenvironment can indeed promote the production of cytokines (Kim and Luster, 2015b). Ligation of Fcγ receptors, pattern recognition receptors, Toll-like receptors, chemoattractant receptors, and complement receptors have all been shown to elicit cytokine expression (O'shea *et al*., 2008). The cytokines being produced by neutrophils exert multiple effects including the regulation of inflammation, angiogenesis, chemotaxis, and so on. The list of cytokines produced by human neutrophils is summarized in Table 2 (Tamassia *et al*., 2018).

Anti-inflammatory cytokines	IL-1ra; TGFβ1; TGFβ2

Proinflammatory cytokines	IL-1α; IL-1β; IL-6; IL-18; Il-22; G-CSF; MIF
C-C chemokines	CCL2; CCL3; CCL4; CCL17; CCL18; CCL19; CCL20; CCL22; CCL23
C-X-C chemokines	CXCL1; CXCL2; CXCL3; CXCL5; CXCL6; CXCL8; CXCL9; CXCL10; CXCL11
Angiogenic and fibrogenic factors	angiopoietin1; Bv8; FGF2; HB-EGF; HGF TGFα; VEGF
Immunoregulatory cytokines	IL-12p40; IL-21; IL-23
TNF family members	APRIL; BAFF; CD30L; CD40L; FasL; RANKL; lymphotoxinβ; TNFα; TRAIL
Other cytokines	activin A; amphiregulin; endothelin; midkine; oncostatin M; PBEF

Table 2. **List of cytokines reproducibly shown to be produced by human neutrophils** (adapted from Tamassia *et al*., 2018 , authorization requested ref # 4657831121245)

Chemokines, mostly belonging to CXC and CC families, are of major importance for coordinating innate and adaptive immune responses, mobilizing neutrophils, monocytes, DCs, NK cells and T helper cells, and orchestrating their sequential recruitment to inflammatory sites (Tecchio and Cassatella, 2016; Yang *et al.*, 2017). Among pro- and anti-inflammatory cytokines, the interleukin family is important in fine-tuning the inflammatory and immune responses. The tumor necrosis (TNF) family, including TRAIL, FasL and BAFF, is also critical for adequate inflammatory and immune processes. The timely production of vascular endothelial growth factor (VEGF) makes neutrophils important in the context of an angiogenetic switch. Colony stimulating factors like G-CSF are powerful signals sparking additional production of immune cells in the bone marrow, and modulating the function of myeloid cells (Meda *et al.*, 1994; Scapini and Cassatella, 2014; Tamassia *et al.*, 2018; Tecchio and Cassatella, 2016; Tecchio *et al.*, 2014).

Cytokine production is highly regulated on multiple levels. Signaling pathways that control various cytokine production in human neutrophils will be presented in a further section.

3.3.2.1. Cytokine signaling

In human neutrophils, the main upstream intermediates that control cytokine production are kinases such as TAK1, p38 MAPK, MEK/ERK, Syk/Src and PI3K/Akt, and transcription factors such as NF-κB, C/EBP and CREB. The involvement of these signaling molecules in cytokine generation will be described in more detail below.

TAK1 (or MAP3K7)

Initially, this kinase was identified as a TGFβ-activated kinase (Yamaguchi *et al*., 1995), thus its name. As a member of the MAP3K family, TAK1 is unique in that its activity requires associated proteins called TAB1 (TAK1 associated protein 1), TAB2, TAB3 (Ishitani *et al.*, 2003; Kanayama *et al.*, 2004; Shibuya *et al.*, 1996; Takaesu *et al.*, 2000) or TAB4 (Prickett *et al.*, 2008). Thus TAB1 and TAB4 directly bind TAK1 and induce it transautophosphorylation *in vitro*. Moreover TAB2 and TAB3 also activate TAK1 when overexpressed. However, unlike TAB1 or TAB4, the TAB2 and TAB3 proteins do not directly activate TAK1 *in vitro*. In contrast, activation of the TAK1 / TAB2 (or TAB3) complex requires an E1-catalyzed ubiquitination reaction (a ubiquitin activating enzyme), Ubc13 / Uev1A (E2: a ubiquitin-conjugating enzyme) and TRAF6 (E3: a ligase ubiquitin) (Wang *et al*., 2001).

In neutrophils TAK1, TAB1, TAB2, and TAB4 are constitutively associated with IκB kinase (IKK) α/β subunits and are expressed both in the nucleus and cytoplasm. Activation of the TAK1 complex in response to inflammatory mediators (TNFα, lpopolysaccharide (LPS)) induces subsequent phosphorylation of the IKK complex, leading to the activation of transcription factor NF-κB. Additionally, NF-κB activation requires phosphorylation of nuclear factor of kappa light polypeptide gene enchancer in B-cells inhibitor (IκB) and its subsequent degradation by the 26S proteasome (Ear *et al.*, 2010; Kravtsova-Ivantsiv and Ciechanover, 2015).

It was shown that TAK1 activation is vital for neutrophil cytokine production in response to TNFα, LPS, fMLP, and GM-CSF. Thus, specific inhibition of TAK1 with 5Z-7-oxozeaenol prevented both mRNA accumulation and protein release of CXCL8, CCL4, and CCL3 in LPS- and TNFα -activated neutrophils (Ear *et al.*, 2010). Additionally, it was shown that neutrophils secrete TNFα in response to LPS activation. Both LPS-induced TNFα mRNA expression and protein secretion were inhibited by 5Z-7-oxozeaenol (Ear *et al*., 2010). More evidence was obtained showing the critical role of TAK1 in neutrophil cytokine secretion. Inhibition of TAK1 prevented IL1-ra and CXCL8 mRNA accumulation and protein secretion by fMLP- and GM-CSF-activated neutrophils. Blocking of TAK1 kinase also intercepted GM-CSF-induced CCL4 mRNA production and protein release (Sylvain-Prévost *et al.*, 2015).

All of these lines of evidence confirm the critical role of TAK1 in neutrophil cytokine generation in response to various stimuli.

MAP kinases (p38 MAPK, MEK/ERK)

Sequential activation of protein kinases is a common signal transduction phenomenon in several cell types (Campbell *et al.*, 1995). Each MAPK cascade is activated either by a small GTP-binding G protein or an adaptor protein, which transmits the signal directly to MAPK kinase kinase (MAP3K). Subsequently, the signal is transmitted downstream of the cascade, MAP2K, MAPK and MAPK-activated protein kinases (MAPKAPK) (Chang and Karin, 2001; L'Allemain, 1994; Tanoue and Nishida, 2002). It is important to note that activation of MAP kinases requires phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) (Brancho *et al*., 2003). Thus, MAPKs phosphorylation sites are threonine (Thr) and tyrosine (Tyr), the middle group is used to distinguish between different MAPKs (Payne *et al.*, 1991).

It was shown that in human neutrophils, MAP kinases p38 and MEK/ERK are activated in response to physiological stimuli (LPS, TNFα, fMLP, GM-CSF, *etc.*) (Coxon *et al*., 2003; McLeish *et al.*, 1998; Nahas *et al.*, 1996; Zu *et al.*, 1998). Ear *et al*. (2010) demonstrated that in response to LPS and TNFα, phosphorylation of p38 MAPK and MEK/ERK and JNK were abolished by TAK1 inhibition, indicating that TAK1 acts upstream of all three MAPKs in LPS- and TNFα-activated neutrophils. Additionally, inhibitors of the p38 MAPK and MEK/ERK pathways substantially diminished the release of CXCL8, CCL4, CCL3 cytokines in neutrophils stimulated with LPS or TNFα; p38 MAPK inhibitor SB203580 also significantly decreased TNFα secretion by LPS-activated neutrophils (Figure 3) (Cloutier *et al.*, 2007).

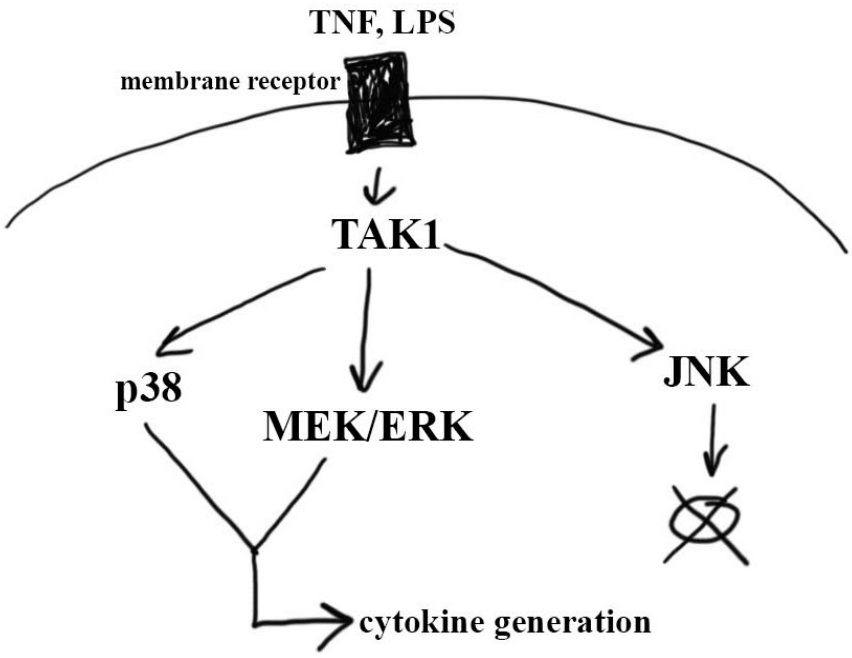

Figure 3. Schematic representation of MAPK pathways in human neutrophils

In response to LPS and TNFα, phosphorylation of p38 MAPK and MEK/ERK and JNK were abolished by TAK1 inhibition. Inhibitors of the p38 MAPK and MEK/ERK pathways substantially diminished the release of CXCL8, CCL4, CCL3 cytokines in neutrophils stimulated with LPS or TNFα, while JNK inhibition did not effect cytokine production (Ear *et al*., 2010, Cloutier *et al.*, 2007).

In neutrophils p38 MAPK and MEK/ERK were also activated in response to fMLP and GM-CSF. However, only ERK phosphorylation is profoundly affected by TAK1 inhibition in response to fMLP and GM-CSF, indicating that TAK1 acts upstream of MEK/ERK but not p38 MAPK in fMLP- and GM-CSF-activated neutrophils. Finally, it was shown that MEK/ERK inhibitor (PD98059) blocks neutrophil secretion of CXCL8, CCL4, and IL-1ra in response to GM-CSF and fMLP, while p38 inhibitors (SB203580, SB202190) had no effect (Sylvain-Prévost *et al.*, 2015).

In summary, both act downstream of TAK1 and participate in cytokine secretion in LPS- and TNFα-activated neutrophils. However, in response to chemoattractants or growth factors,

TAK1 mainly acts through the MEK/ERK pathway. Likewise, MEK/ERK kinases but not p38 MAPK are involved in fMLP and GM-CSF induced cytokine release in human neutrophils

PI3K/Akt

PI3K is a heterodimeric enzyme composed of a regulatory and catalytic subunit. The PI3K family is complex and can be divided into three sub-families (classes: I, II, and III), taking into account their structural characteristics, their activation mechanisms, and the substrate specificity of their catalytic subunit. However, regardless of their class, all PI3Ks share a common substrate: phosphatidylinositol (PI). The enzymatic reaction catalyzed by PI3K leads to the formation of phosphorylated lipids: PI → PI 3-phosphate, (PI(4)P) → PI 3,4-bisphosphate, (PI(4,5)P2) → PI 3,4,5-triphosphate (PI(3,4,5)P3) (Hawkins and Stephens, 2015). These phosphorylated lipids work as second messengers, subsequently binding intracellular proteins containing a pleckstrin homology (PH) domain. Akt is a Ser/Thr kinase containing a PH domain. Upon PI3K activation, Akt translocates to the membrane where it binds PI(3,4,5)P3. This binding induces conformational changes that allow Akt activation by other kinases. Akt has two phosphorylation sites that are essential for kinase activation, Thr^{308} in the kinase domain and Ser^{473} in the C-terminal domain (Alessi and Cohen, 1998; Kandel and Hay, 1999; Manning and Toker, 2017).

It was shown that the PI3K/Akt pathway is implicated in pro-inflammatory cytokine production by human neutrophils. Indeed, inhibition of LPS- and TNFα-activated neutrophils with LY294002 (a pan-PI3K inhibitor) vastly decreased CCL3, CCL4, and CXCL8 secretion

(Fortin *et al.*, 2011). Likewise, inhibition of PI3K significantly decreased cytokine (CCL4 and CXCL8) release by fMLP and GM-CSF activated neutrophils. Additionally, significant inhibition of IL-ra secretion by LY294002 was observed in GM-CSF-activated neutrophils (Sylvain-Prévost *et al.*, 2015). Cross intercepted talk between Akt and TAK1 was investigated. Thus it was shown that in human neutrophils, Akt acts downstream of TAK1 in response to LPS and TNFα, while in GM-CSF- and fMLP-activated cells, TAK1 does not affect Akt phosphorylation (Fortin *et al.*, 2011; Sylvain-Prévost *et al.*, 2015).

Together, this evidence indicates an important role of PI3K/Akt pathway in cytokine secretion by neutrophils activated with physiological stimuli.

Syk/Src

Both Syk and Src family kinases belong to the family of non-receptor tyrosine kinases (NRTKs). These enzymes catalyze the transfer of phosphate groups to tyrosine residues on protein substrates. Substrate phosphorylation causes changes in their function and/or enzymatic activity, leading to specific biological responses including cell differentiation, proliferation, adhesion, survival, etc. (Gocek *et al.*, 2014; Mócsai *et al.*, 2010; Roskoski, 2004; Tsang *et al.*, 2008; Turner *et al*., 2000; Varmus *et al.*, 1989). The activity of NRTKs is tightly regulated, as summarized below.

All members of Src tyrosine kinases family contain a 14-carbon myristoyl group attached to a Src homology (SH)-4 domain, a SH3 domain, a SH2 domain, a SH2-kinase linker, a protein–tyrosine kinase domain (the SH1 domain) and a C-terminal regulatory segment that

has an auto-inhibitory phosphorylation site (Tyr-530) (Roskoski, 2005). Under resting conditions, Src is phosphorylated at Tyr-530; this restrains the enzyme and prevents accessibility of the SH2 and SH3 domains to external ligands. Activation of Src kinase starts with the dephosphorylation of Tyr-530, which subsequently leads to conformational changes and an intermolecular autophosphorylation at tyrosine 419 (Tyr-419); this residue is present in the activation loop, and its phosphorylation promotes kinase activity (Brickell, 1992; Gocek *et al.*, 2014; Roskoski, 2005; Superti-Furga and Courtneidge, 1995; Varmus *et al.*, 1989).

The Syk structure consists of a C-terminal kinase domain, a N-terminal pair of SH2 domains separated by an inter-SH2 linker, and a SH2-domain-kinase linker. Unlike Src kinase, Syk has a second SH2 instead of an SH3 domain. Recruitment of Syk to immune receptors involves binding of the tandem SH2 domains of Syk to motifs in the receptor known as immune tyrosine activation motifs (ITAMs). Binding of Syk kinase to phosphorylated ITAMs results in conformational changes that disrupt its autoinhibited structure, thus allowing kinase activation. After activation, Syk is tyrosine phosphorylated by trans- or autophosphorylation. Thus Syk have multiple phosphorylation sites that regulate its activity and serve as docking motifs for other proteins. These sites include Tyr-348 and Tyr-352 within the SH2-linker region, Tyr-525 and Tyr-526 within the activation loop of the kinase domain, Tyr-630 in the C terminus of Syk, and other sites (de Castro, 2011; Gocek *et al.*, 2014; Mócsai *et al.*, 2010; Tohyama and Yamamura, 2009; Tsang *et al.*, 2008).

Despite the important role of NRTKs in various immune responses (Bjorge *et al*., 2000; Mócsai *et al*., 2010; Turner *et al*., 2000; Varmus *et al*., 1989), their involvement in cytokine

production by neutrophils was elusive until recently. In this respect, we reported that Src or Syk inhibition strongly represses the secretion of CXCL8 induced by physiological neutrophil agonists (i.e., LPS, TNFα, fMLP, and GM-CSF) (Cloutier *et al*., 2009a; Ear *et al.*, 2010, Cloutier *et al.*, 2009b; Mayer *et al.*, 2013). However, TNF induced CXCL8 release in a Syk-independent manner. In addition, CCL4 secretion was significantly prevented by Src and Syk inhibitors in fMLP- and GM-CSF-activated neutrophils, but was only moderately affected in LPS- or TNF-stimulated cells. These results indicate that Src or Syk inflammatory cytokine control is largely dependent on the stimulus. We also revised the mechanism by which these tyrosine kinases act on cytokine generation (Ear *et al.*, 2017). Thus, the induction of inflammatory cytokine gene expression was unaffected by Src or Syk inhibition, suggesting that they act posttranscriptionally. Accordingly, several signaling intermediates known to affect cytokine translation (MNK1; ribosomal S6 kinase and its substrate, the S6 ribosomal protein; and to a lesser extent, of 4E-BP1) were found to be under the control of Src and Syk (Ear *et al.*, 2017). Among these downstream targets, MNK1 is particularly relevant, as it was shown previously to participate in the translational regulation of cytokine generation in neutrophils (Fortin *et al.*, 2013). In summary, results obtained indicate that Src and Syk control inflammatory cytokine generation at the translational level in primary neutrophils (Ear *et al.*, 2017).

NF-κB, C/EBPβ, CREB transcription factors

Transcription factors (TF) are commonly composed of a DNA-binding domain, an activation domain and dimerization domain. The DNA-binding domain allows the attachment

to target sequences within target gene promoters. The activation domain is typically the target of post-translational modifications, such as phosphorylation and acetylation, altering the ability to interact with co-activators, co-repressors or proteins of the transcriptional machinery. The co-activators, co-repressors and co-regulators generally do not have a DNA binding domain and therefore need to interact with the transcription factors to perform their functions. Interactions between TF and co-activators upregulate the transactivations of target genes (Aerts, 2012; Latchman, 1993). Also TFs can work as repressors, blocking the recruitment of RNA polymerase to specific genes (Nikolov and Burley, 1997; Owen-Hughes and Workman, 1994).

There are approximately 2600 proteins in the human genome that contain a DNA binding domain and are therefore potential transcription factors involved in the regulation some 20,000 to 30,000 genes (Babu *et al.*, 2004). The study of the cytokine and chemokine promoter sequences revealed the importance of several families of transcription factors: NF-κB (Nuclear factor - kappa B), C/EBP (CCAAT enhancer binding protein) and CREB (cAMP response element binding protein) (Escoubet-Lozach *et al.*, 2002; Holloway *et al.*, 2002; Nikolajczyk, 2006; Tsuruta *et al.*, 1998), are among them. It was shown that in neutrophils, NF-κB, CREB1 and C/EBPβ play important roles in cytokine production, at least in response to LPS and TNFα. Additionally, upstream events were elucidated. Namely, both CREB1 and C/EBPβ act downstream of p38 MAPK and MSK1, while NF-κB acts downstream of IKK and TAK1 (Cloutier *et al.*, 2009a; Ear *et al.*, 2010). The PLB-985 cell line was used to investigate the role of NF-κB, CREB1 and C/EBPβ in cytokine production. It was shown that DMSO-differentiated PLB-985 cells have neutrophil-like transcription factor activation

profile, as well as cytokine production profile. Thus, overexpression of dominant-negative IκB-α [IκBα (S32,36A)] in differentiated PLB-985 cells significantly reduced the production of CXCL8, CCL4, and CCL3 in response to LPS and TNFα. These results indicate an important role of the NF-κB transcription factor in cytokine production by human granulocytes (Ear and McDonald, 2008). The same method was applied to investigate the role of CREB1 and C/EBPβ in cytokine generation. Overexpression of dominant-negative C/EBPβ (A-CEBP2N3T) and CREB1 (K-CREB) in neutrophil-like PLB-985 cells vastly inhibited CXCL8, CCL3 and CCL4 release in response to LPS and TNFα (Cloutier *et al.*, 2009b; Mayer *et al.*, 2013). Also, the induced generation of IL-1β and TNFα was significantly inhibited by K-CREB overexpression in differentiated PLB-985 cells (Mayer *et al.*, 2013). In summary, the above results unveil the vital role of NF-κB, CREB1 and C/EBPβ in cytokine production by human granulocytes.

3.4. NETs

3.4.1. History

Early studies found that neutrophil stimulation with the potent PKC activator, phorbol 12-myristate 13-acetate (PMA), initiated a unique form of cell death that is morphologically distinct from other known forms – necroptosis, apoptosis, autophagy, etc (Takei *et al.*, 1996). Stimulation with PMA was observed to decrease chromatin density and to induce nuclear membrane degeneration. Stimulation for 3 hours increased cell membrane permeability, with cell death peaking after 4 hours incubation. Agarose and pulsed-field gel electrophoresis analysis confirmed that DNA had not been degraded (Takei *et al.*, 1996), demonstrating that PMA induces DNA release.

Almost 10 years later, in 2004, Brinkman *et al*. demonstrated that neutrophils can form extracellular fibers that consist of chromatin associated with granular and nuclear proteins, which they named neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004b). Immunohistochemistry analyses revealed that NETs contained proteins from azurophilic (primary) granules such as neutrophil elastase, cathepsin G, and myeloperoxidase (Figure 4). Proteins from specific (secondary) granules and tertiary granules, such as lactoferrin and gelatinase, respectively, were also present. This group further postulated that DNA is a major structural component of NETs not only because all the DNA intercalating dyes that were tested strongly labeled NETs, but also because treatment with deoxyribonuclease (DNase) resulted in complete disintegration of the NETs. On the other hand, protease treatment left extracellular chromatin filaments intact. They showed that NETs reacted with antibodies against various histones (H1, H2A, H2B, H3, H4) and against the H2A-H2B DNA complex (Brinkmann *et al.*, 2004). Likewise, they confirmed that neutrophils were actively forming NETs in response to various stimuli such as IL-8, LPS, or PMA. Later studies from this group found that NETosis represents a form of cell death distinct from apoptosis and necrosis (Fuchs *et al.*, 2007), supporting earlier work by Takei and colleagues.

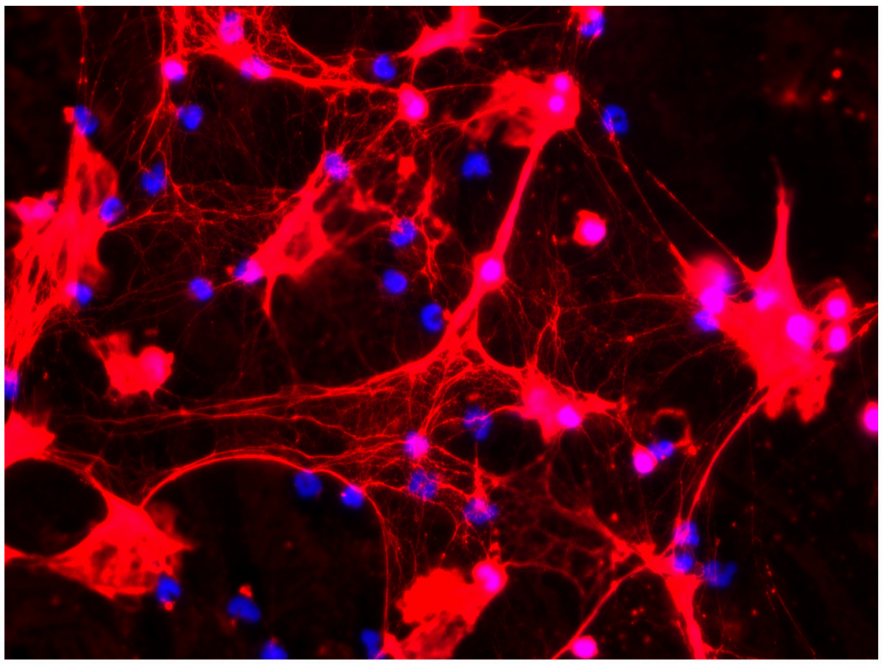

Figure 4. Representative image of NETs induced *in vitro* by fMLP in human neutrophils.

NETs are visualized by costaining of myeloperoxidase (red) and nuclear material (DAPI, blue). Magnification is 40X. (Tatsiy, unpublished)

More importantly, studies from Brinkman *et al*., demonstrated physiological functionality of the phenomenon, they showed that after induction, 'sticky' extracellular chromatin filaments trapped bacteria, both gram-positive and gram-negative, and successfully killed them (Brinkmann *et al.*, 2004). Following this study, NETs were recognized as a *bona fide* neutrophil response that physically prevents spread of bacteria, increases effectiveness of neutrophil antimicrobial substances by ensuring there high local concentration, and assists in killing bacteria.

3.4.1.1. Vital and suicidal NETs

Exploration of the mechanisms underlying NETosis highlighted different forms of NETosis (Yipp and Kubes, 2013). Several studies have suggested that NET formation may not always be associated with cellular membrane disruption, but could also be performed by cells that remain intact after DNA extrusion. This version of NETosis was called vital. After completing vital NETosis neutrophils could retain other host defense functions, such as migration and phagocytosis. During vital NETosis, DNA release from the nucleus occurs by packing of DNA in budding vesicles derived from the nuclear membrane, which subsequently exocytose without cellular membrane rupture (Figure 5) (Clark *et al*., 2007; Pilsczek *et al*., 2010; Yipp *et al*., 2012a). This process was described to be rapid, occurring within 30-60 min, and induced by various stimuli (Byrd *et al*., 2013; Yipp *et al*., 2012a).

In contrast, lytic NET release begins after 2 h and usually goes on for 4 – 6 h (Steinberg and Grinstein, 2007). Lytic NET formation or so-called suicidal NETosis is characterized by nuclear chromatin decondensation that is followed by nuclear and cellular membrane rupture (Brinkmann *et al*., 2004; Fuchs *et al*., 2007; Steinberg and Grinstein, 2007). Even though there is no clear understanding if these two types of NET formation are interconnected, it was agreed that mechanisms of vital and suicidal NET formation have to be divided and investigated separately (Yipp and Kubes, 2013). My further narrative along with work established in this thesis is focused on understanding signaling pathways and mechanisms that control suicidal NET formation.

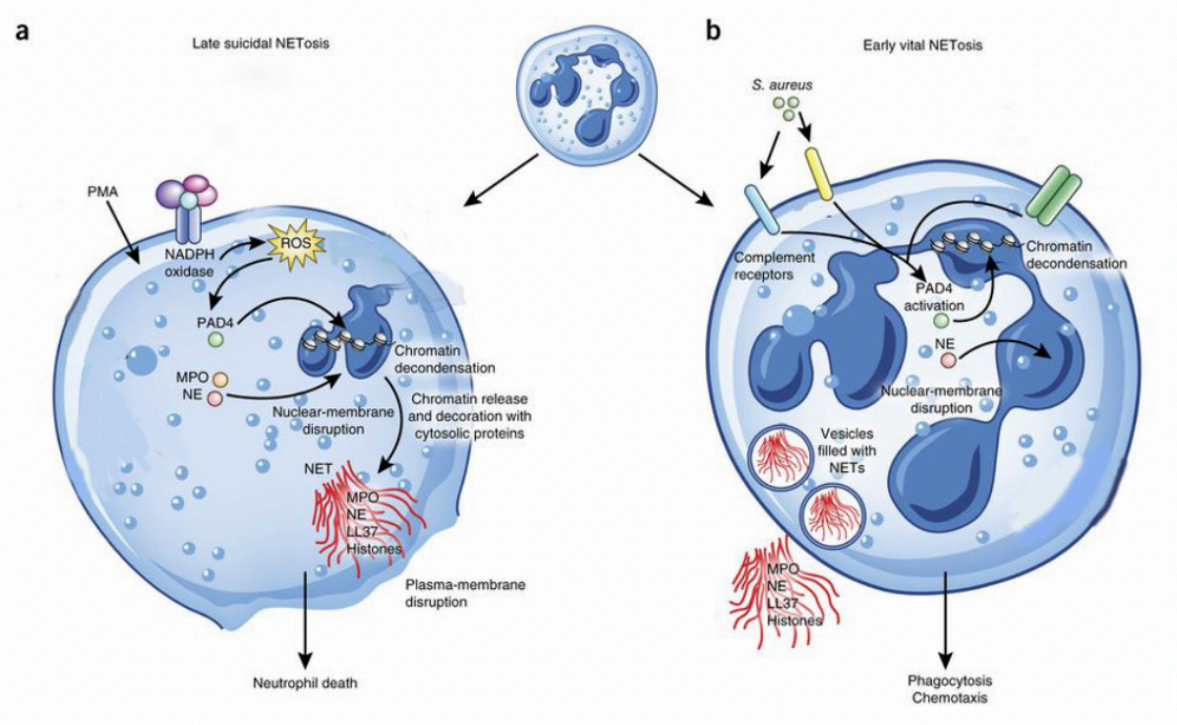

Figure 5.Overview of NETosis.

(**a**) Different stimuli, including PMA induce suicidal NETosis, which occurs after hours of stimulation. In response to PMA NADPH oxidase is activated, ROS are produced and PAD4 is activated, which results in chromatin decondensation. Afterward, neutrophil elastase (NE) and myeloperoxidase (MPO) are translocated into the nucleus to promote further unfolding of chromatin, with resultant disruption of the nuclear membrane. Chromatin is released into the cytosol, where it becomes decorated with granular and cytosolic proteins. Finally, NETs are released through disruption of the plasma membrane, and the neutrophil dies. (**b**) Vital NETosis is induced within minutes by *S. aureus* and other stimuli. PAD4 is activated and induces chromatin decondensation. Like in suicidal NETosis, NE is translocated into the nucleus to promote further chromatin decondensation and nuclear-membrane disruption. However, protein-decorated chromatin is expelled via vesicles, and the neutrophil stays alive for further functions. (adapted from Jorch and Kubes, 2017, authorization requested ref # 4657390257467)

3.4.2. NETosis as a functional response

Distinguishing NETosis as a true neutrophil functional response (as opposed to just another type of cell death) was instrumental in prompting investigations to understand NET formation

mechanisms. In the pioneering report on NET formation (Brinkmann *et al*., 2004b), it was proposed that histone-rich chromatin filaments are released from intact, viable neutrophils. This was mainly based on the following observations: no cytosolic proteins were detected on the DNA filaments extruded from the cells; most cells excluded vital dyes; and NETs were detected within 30-60 min after stimulation with compounds known to prolong neutrophil lifespan, such as IL-8 and LPS (Fox *et al*., 2010; Remijsen *et al*., 2011). However, the same group later demonstrated that cellular membranes rupture during NETosis (Fuchs *et al*., 2007) was a distinguishing feature in comparison to apoptosis (Zhang *et al*., 2018). Similar observations were also reported by another group (Steinberg and Grinstein, 2007). Thus, it came to be encountered that in contrast to apoptosis, neutrophils undergoing NETosis did not display phosphatidylserine (PS) on their surface before plasma membrane disruption, preventing their clearance by phagocytes (Remijsen *et al*., 2011a; Segawa and Nagata, 2015). Likewise, in contrast to apoptosis or programmed necrosis, during NET formation both the nuclear and granular membranes disintegrate and granular content mixes with nuclear chromatin (Fuchs *et al*., 2007). Finally, NETosis does not feature the main morphological signs of apoptosis, such as membrane blebbing, nuclear chromatin condensation, PS exposure and internucleosomal DNA cleavage before plasma membrane rupture (Fuchs *et al*., 2007; Luo and Loison, 2008; Remijsen *et al*., 2011). Also, several lines of evidence were obtained, supporting that the tightly controlled mechanisms driving NETosis set it apart from those of necrosis or apoptosis. In particular, caspase activity, which is a crucial indicator of both necrosis and apoptosis, was not detected during neutrophils extracellular filament formation (Luo and Loison, 2008; Remijsen *et al*., 2011; Röhm *et al*., 2014). Moreover, necrotic neutrophils stain positive for F-actin, but not after undergoing NETosis (Marcos *et al*., 2010;

Ramos-Kichik *et al*., 2009; Remijsen *et al*., 2011). Collectively, the above evidence show that NETosis is a tightly controlled cellar process in its own right.

Since the first description of NETs in 2004, our knowledge about this phenomenon has dramatically expanded, but there remain significant gaps in our understanding of the underlying mechanisms and upstream signaling. It is widely agreed now, that two major events precede NET formation: first is chromatin decondensation and association with antimicrobial proteins (Fuchs *et al*., 2007), second is chromatin extrusion (Neubert *et al*., 2018a). Several studies focused on the mechanism of chromatin decondensation, but very little is known about the mechanisms that control chromatin extrusion.

3.4.2.1. The first major step of NETosis: chromatin decondensation

It is important to mention that all the data described below, except for those relating to 'citrullination,' were obtained using PMA as a stimulus to induce NETosis. This particular compound is a PKC activator (Chang and Beezhold, 1993) that has been widely used because of its affordability and high potency to induce NET formation.

3.4.2.1.1. Neutrophil elastase (NE) and myeloperoxidase (MPO) promote chromatin decondensation

In 2010 the same group that initially described NETs proposed a model of chromatin decondensation (Papayannopoulos *et al*., 2010). They demonstrated that NE is essential to

initiate NET formation and that in cooperation with MPO, it drives chromatin decondensation. To this end, they developed a cell-free nuclear decondensation assay using intact nuclei and cytoplasmic extracts from neutrophils. Incubation of intact nuclei with various concentrations of MPO led to chromatin decondensation. Interestingly, this process did not depend on MPO activity. Likewise, incubation of intact nuclei with NE led to chromatin decondensation and was prevented by the addition of a NE inhibitor (NEi). Addition of the two proteases to the intact nuclei accelerated decondensation, showing that both proteins synergize. Then using western blot (WB) analysis, they confirmed that NE partially degrades core histones during PMA-induced NET formation and that this process can be reversed by NEi (Papayannopoulos *et al*., 2010). Summarizing these data, the following model was proposed.

Upon activation, NE somehow escapes the granules and translocates to the nucleus, where it cleaves histones and thereby promotes chromatin decondensation. In addition, MPO similarly escapes cytoplasmic granules, enters the nucleus, and binds to chromatin in the late stages of the process to promote further chromatin decondensation (Papayannopoulos *et al.*, 2010).

In subsequent studies, the same authors added some details to the above model. They described the mechanism of ROS-mediated NE translocation to the nucleus. Using an anti-NE antibody, they isolated a new complex that they named “azurosome”. This complex contained NE, cathepsin G (CG), azurocidin (AZU), MPO, lactoferrin (LTF), proteinase 3 (PR3), and lysozyme (LYZ). In resting neutrophils, the azurosome was associated with a subset of azurophilic granule membranes. Upon activation of the oxidase complex, ROS such

as H_2O_2 trigger the activation and dissociation of NE/CG/AZU from the granules and release into the cytoplasm. The dissociated part of the azurosome binds to F-actin and degrades it, which allows active NE to enter the nucleus. Notably, that mechanism of controlled granule component release does not seem to involve membrane fusion, but the exact mechanism of NE release remains unknown (Metzler *et al*., 2014).

3.4.2.1.2. LL37 mediates disruption of the nuclear membrane during NET formation

The antimicrobial peptide, LL37, a member of the α-helical cathelicidins, binds and damages bacterial membranes that are deficient in cholesterol and sphingomyelin (Xhindoli *et al.*, 2016). Increased levels of LL37 are found in inflamed or infected tissues where it can exert direct microbicidal activity against not only bacteria but also fungi and enveloped viruses (Alalwani *et al.*, 2010; Kahlenberg and Kaplan, 2013; Tjabringa *et al.*, 2003). During their maturation, neutrophils produce and store LL37 in their secondary neutrophilic granules. In response to stimuli such as IL-8, neutrophils can release LL37 (Dürr *et al*., 2006). Conversely, LL37 can chemoattract neutrophils, thus recruiting even more leukocytes to sites of injury or infection, while increasing the local concentration of pro-inflammatory mediators (De Yang *et al.*, 2000). Immunofluorescence microscopy analyses revealed that addition of LL37 to PMA-activated neutrophils dramatically increases NET formation (Neumann *et al.*, 2014a). Moreover, LL37 alone can induce nuclear rupture and subsequent neutrophil extracellular trap formation (Neumann *et al.*, 2014b, 2014a). Additional immunohistochemistry assays showed that externally added LL-37 translocates towards the nucleus and co-localizes to the nucleus

membrane. This leads to the loss of nuclear membrane receptor signal - lamin B during the process of NET formation (Neumann *et al.*, 2014a). Collectively, these data support the involvement of LL37 in nuclear membrane rupture.

3.4.2.2. The second major step of NETosis: chromatin extrusion

It was proposed that cellular membrane rupture during NETosis is driven by material properties of swollen chromatin, escaped from the ruptured nuclear envelope. Proposers of this hypothesis even noticed a correlation between time of chromatin extrusion and neutrophil size. They showed that smaller neutrophils form NETs faster than larger size neutrophils (Neubert *et al.*, 2018b). However, this hypothesis was based on the experiments performed with PMA-activated neutrophils. In addition, it was demonstrated that different stimuli induce different combinations of NET types (Hakkim *et al.*, 2011). PMA, predominantly induces diffuse NETs that are characterized by leaked chromatin forming a diffused “cloud” around the cellular membrane. On the other hand, many physiological stimuli were observed to form NETs featuring a meshwork of long filaments, which some refer to as spread NETs (Hakkim *et al.*, 2011). To date, no explanation has been put forward to clarify the mechanism of long filament formation.

3.4.3. The role of endogenous ROS

A link between ROS production and NET formation was initially postulated after examination the ability of neutrophils derived from patients with chronic granulomatous

disease (CGD) to form NETs. CGD patients have a defective NADPH oxidase complex, which renders them incapable of forming ROS. Neutrophils from CGD patients did not undergo NETosis in response to PMA or *S. aureus* ingestion (Fuchs *et al.*, 2007). Likewise, the NADPH oxidase inhibitor, DPI, prevents NET formation in neutrophils derived from healthy donors in response to PMA and some bacteria. Additionally, it was shown that some ROS (*e.g.*, singlet oxygen, HOCl, H_2O_2) can directly induce NETs (Akong-Moore *et al.*, 2012; Nishinaka *et al.*, 2011; Palmer *et al.*, 2012). Together, these findings led to the widespread view that NET formation is a ROS-dependent process (Björnsdottir *et al.*, 2015; Stoiber *et al.*, 2015).

However, most of the studies of NETosis were performed using PMA (a powerful NADPH oxidase activator) as a stimulus (Karlsson *et al.*, 2000). Conversely, many stimuli that induce little or no ROS (*e.g.*, IL1β, TNFα, GM-CSF) were found to be NET inducers (Pang *et al.*, 2013a; Parker *et al.*, 2012a). Over time, more evidence were obtained regarding various physiological stimuli (poor ROS inducers (Nguyen et al., 2017)) that can potently induce NETs (Kaplan and Radic, 2012). The actual role of the NADPH oxidase in NET formation therefore deserves to be elucidated, and this particular point will be investigated in this thesis.

3.4.4. The role of PAD4 and citrullination

Activation of neutrophils with pro-inflammatory stimuli strongly promotes histone citrullination (Neeli *et al.*, 2008; Wang *et al.*, 2009a). Citrullination is the conversion of positively charged arginine side chains into polar but uncharged citrulline side chains, by

deimination (Figure 6). This process therefore decreases the ability of histones to interact with negatively-charged DNA (Bicker and Thompson, 2013). There are five human peptidyl arginine deiminases (PADs) known to catalyze such conversions, with PAD4 being the most extensively studied isoform. PAD4 enzyme activation is calcium-dependent. Calcium binding induces significant conformational changes that are important for catalytic activity (Wang and Wang, 2013). PAD4 is expressed in various leukocytes and is especially abundant in neutrophils (Jones *et al.*, 2009). PAD4 is the only member of the PAD family to feature a nuclear localization sequence and as a result, it mainly localizes to the nucleus, where it targets histones H2A, H3, and H4 (Wang *et al.*, 2009). However, it is important to note that PAD2, another member of the PAD family that is expressed in neutrophils, is also known to localize to the nucleus (Zhou *et al.*, 2017).

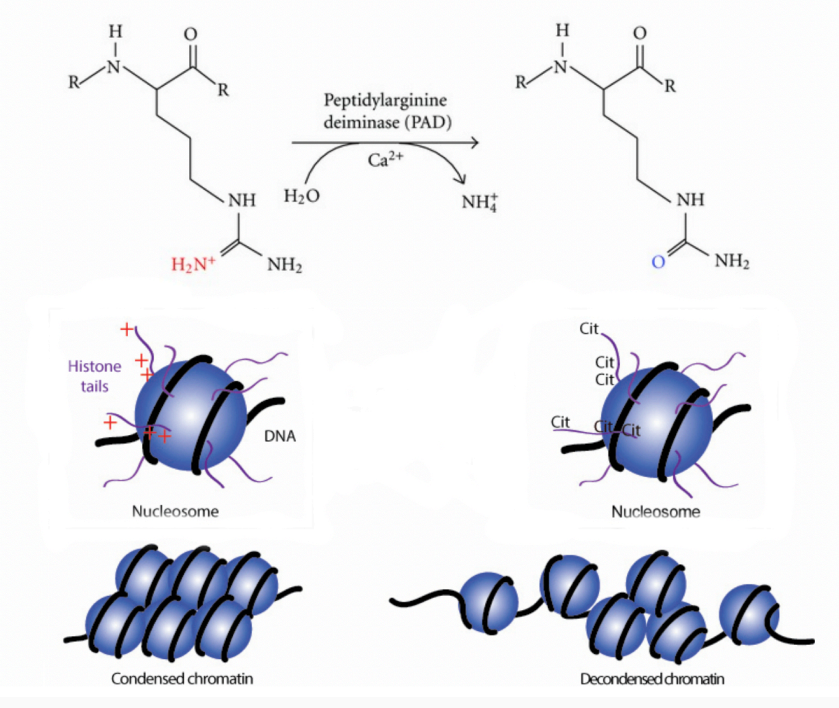

Figure 6. Peptidyl arginine deiminas (PAD) enzymes catalyze the conversion of protein arginine residues to citrulline.

This process decreases the ability of histones to interact with negatively-charged DNA and leads to chromatin decondensation (adapted from Mohanan *et al*., 2012, distributed under the Creative Commons Attribution License)

Data from several groups supports the importance of PAD4-catalyzed histone hypercitrullination in chromatin decondensation during NETosis. Immunofluorescence analyses of differentiated HL60 neutrophil-like cells demonstrated that before treatment with calcium ionophore, nuclei showed a clear multilobular structure, while after treatment, nuclei became round; this process was found to be tightly coupled with an increase in H4Cit staining (Wang *et al.*, 2009). Incubation of the cells with Cl-amidine, a potent pan-PAD inhibitor, partially prevented nuclear metamorphosis (Wang *et al.*, 2009). Additionally, overexpression of PAD4 in osteosarcoma U2OS cells, which are incapable of forming NETs, induced apoptosis-independent chromatin decondensation and the formation of NET-like structures (Leshner *et al.*, 2012). Furthermore, neutrophils isolated from PAD4-knockout mice demonstrated complete inhibition or impaired NET formation, depending on the conditions (Li *et al.*, 2010). Together these data suggest an important role for PAD4 during the decondensation step of NETosis.

Even though PAD4 involvement in NET formation has been proposed in various models, it remains unclear, which PAD isoform is involved in human NET formation. In this context, both PAD2 and PAD4 are expressed in human neutrophils and associated with NETs in response to inflammatory stimuli. The abrogation of NET formation by the pan-PAD inhibitor, Cl-amidine, likewise does not allow one to determine which PAD isoform is involved.

3.4.5. NET signaling

A limited number of studies have addressed the signaling pathways involved in NET formation in response to physiological stimuli. It is known that stimuli of different origin such as cytokines (IL8, TNFα), TLR ligands (LPS), chemotactic peptides (fMLP), immobilized immune complexes (iIC), and polysaccharides (β-glucan) can induce NET formation. Some singnaling pathways were described to be involved for certain stimuli, but a general picture has not yet emerged.

For instance, it was shown that in response to iIC, neutrophils undergo ROS-dependent NETosis that requires FcγRIIIb. Additionally, iIC-induced phosphorylation of Akt, p38 MAPK, ERK1/2 and activation of PI3K. Inhibition of these signaling molecules disrupted NET formation in response to iIC. Furthermore, upstream signalling events were evaluated. PP2 (pan-Src inhibitor) prevented iIC-induced activation of Akt, p38 MAPK, ERK1/2, PI3K and abolished NET formation. This indicates that Src kinase activation is involved in iIC-induced NETosis and acts upstream of Akt, p38 MAPK, ERK1/2, PI3K. The role of another tyrosine kinase, Syk, was also evaluated. The Syk inhibitor, piceatannol, also blocked NETosis in response to iIC (Behnen *et al.*, 2014).

A study by Carestia et.al (2016) showed that PI3K, p38 MAPK, ERK1/2 and Src are also involved in NET formation induced by LPS. However, levels of inhibition were not as marked as for iIC-induced NETosis. Indeed, p38 MAPK inhibition abolished NET formation in response to LPS, while inhibition of PI3K, ERK1/2 and Src reduced NET formation only by

about 40%. It is worthy of to note that Caresia et.al used a pan-Src inhibitor (PP1); this aspect might be important, since PP1 and PP2 have different specificities for various Src family kinases (Carestia *et al.*, 2016).

More studies investigated the role of PI3K, Syk, TAK1 and Src kinases during NET formation in response to physiological stimuli. Wortmannin, a pan-PI3K inhibitor, prevented NET formation in response to fMLP (Itakura and McCarty, 2013). β-glucan-induced NETs were abolished by both Src and Syk inhibitors (PP2 and PRT-060318, respectively) (Nanì *et al.*, 2015). In addition, it was shown that TAK1 inhibitor, (5Z)-7-Oxozeaenol, significantly decreased FcγRIIIb-induced NET formation (Alemán *et al.*, 2016).

In summary, disparate lines of evidence indicate that TAK1, PI3K/Akt, MAPKs (p38 MAPK and ERK1/2) and Syk/Src signaling pathways are involved in NET formation, at least in response to some physiological stimuli.

3.4.6. The importance of NETs in homeostasis and disease

As professional microbe killers, neutrophils are most often the first line of defense of the innate immune system. Classical neutrophil functional responses include phagocytosis and degranulation, as well as lipid mediator synthesis. In the 1990s, it was shown that neutrophils can also produce a broad spectrum of pro-inflammatory chemokines and cytokines, which influence the inflammatory and immune reactions. More recently, NETosis emerged as another powerful means of defense against different types of pathogens – bacteria, yeasts,

fungi, viruses. A role for NETs has been demonstrated ~~and postulated~~ in infectious diseases and autoimmunity. This section is an overview of the evidence.

3.4.6.1. Insidious traps: NETs in infection

3.4.6.1. Bacterial infections

Some of the first studies to show the biological relevance of NETs were conducted using sepsis patients. *In vitro* and *in vivo* experiments demonstrated trapping and subsequent killing of gram-positive and gram-negative bacteria by NET filaments (Brinkmann *et al*., 2004b; Pilsczek *et al*., 2010; Wartha *et al*., 2007; Yost *et al*., 2009). It was concluded that NETs were contributing to host defense during severe bacterial sepsis. Additionally, NETs were shown to be produced in response to septic stimuli intravascularly. This process was activated by LFA-1-mediated platelet-neutrophil interactions in both murine and human sepsis (McDonald *et al*., 2012). To further investigate the importance of NETs for disease outcome, DNase was perfused, and it abolished NET formation during staphylococcal infection and led to a significantly accelerated release of bacteria from the entry site. This increased presence of bacteria in the bloodstream (bacteremia) and reduced the lifespan of animals. Another element that suggested a role for NETs in sepsis pathogenesis was obtained using NE knockout mice (NE-/-). Elastase is a granular protease that associates with NETs. In NE-/- mice, defective bacterial killing and increased susceptibility to sepsis were observed following *Klebsiella pneumonia* or *Escherichia coli* infection (Belaaouaj *et al.*, 1998; Yipp *et al.*, 2012). More compelling evidence was provided in a later study, which demonstrated that NE-/- mice failed

to produce NETs (Papayannopoulos *et al.*, 2010a). Thus, it has now become established that NETs play an essential role in several bacterial diseases (Hasler *et al.*, 2016; Stephan and Fabri, 2015).

Interestingly, some bacteria have developed countermeasures against the toxic effect of NETs. *Staphylococcus aureus* induces NETs that kill them essentially via the virulence factor Panton-Valentine leukocidin, but to escape, *S. aureus* expresses a nuclease that degrades NETs, thereby enhancing infectivity in a mouse respiratory tract infection model . Other strategies applied by bacteria are summarized in Table 2 (Hasler *et al*., 2016).

Infectious organism	**Mechanism of evasion**	**Effect**
Staphylococcus aureus	Endonuclease	Virulence and evasion of killing
Group A *streptococcus*	Endonuclease	Virulence and evasion of killing
Neisseria gonorhoeae	Thermonuclease	Virulence and evasion of killing
Neisseria meningitidis	Cathepsin G inhibition and bacterial outer membrane vesicle release	Virulence and evasion of killing
Yersinia enterocolitica	Endonuclease	Virulence and evasion of killing
Vibrio cholerae	Endonuclease	Virulence and evasion of killing
Bordetella pertussis	Adenylate cyclase toxin	Inhibition of NET formation

Leptospira species	Endonuclease	Virulence and evasion of killing
Sepsis	CXCR2 and phospholipase D2 activation	Inhibition of NET formation
Mycobacterium tuberculosis	IL-10 adherent to NETs	Reduced macrophage activity

Table 3. Strategies of bacteria directed against neutrophil extracellular traps. (adapted from Hasler *et al*., 2016, distributed under the Creative Commons Attribution License)

3.4.6.1.2. Viral infections

Several viruses have been found to stimulate NET formation including influenza A, human immunodeficiency virus (HIV)-1, hantaviruses, etc. (Schönrich and Raftery, 2016). The mechanism of NET induction by viruses is not entirely understood. However, it was shown that HIV-1 triggers NET formation via the TLR7 and TLR8 receptors. NETs capture and partially inactivate HIV-1 virus. This inactivation can be reversed either by MPO inhibitor or by an anti-defensin antibody, showing an essential role of NET-associated proteins against viral infection (Saitoh *et al.*, 2012). More data were obtained showing antiviral properties of NETs. Histones, which account for the majority of proteins on NETs, showed potent antiviral activity against influenza A viruses. Likewise, influenza viruses often trigger pneumonitis with excessive neutrophil infiltration and NET formation that contribute to severe lung tissue damage (Narasaraju *et al.*, 2011). Respiratory syncytial virus (RSV) also induces NET formation. NETs can capture RSV virions and prevent their attachment to epithelial cells *in vitro* (Cortjens *et al.*, 2016).

Additionally, pathogenic hantaviruses can induce ROS-dependent NET formation and stimulate production of high levels of antinuclear antibodies. However, it is still unknown if these antibodies have an impact on viral replication or NET activity (Schönrich and Raftery, 2016). To summarize, this data shows that NETs play an active role during a viral infection.

3.4.6.1.3. Fungal infections

Fungi are another type of pathogens that trigger NETosis. It was demonstrated that fungi are susceptible to NET-mediated killing. NETs can kill both yeast and hyphal forms of *Candida albicans* (Urban *et al*., 2006). NETs induced by *Candida albicans* were highly dependent on PAD4 activity, as NET formation was abrogated in neutrophils from PAD -/- mice (Urban *et al*., 2009). Furthermore, NETs are induced in response to different morphotypes of *Aspergillus fumigatus*, i.e., resting or swollen conidia and hyphae. NET formation in response to *A. fumigatus* was utterly abolished by NDPH inhibitor, DPI (diphenyliodonium), showing that ROS production is important for NET induction by *A. fumigatus* (Bruns *et al.*, 2010). Also, it was demonstrated that β-glucan receptor signaling is required for *A. fumigatus* NET-mediated inhibition and/or killing *in vitro*. Additionally, *A. fumigatus* developed the escaping NET mechanism. Namely, it was shown that the virulence factor produced by *A. fumigatus*, galactosaminogalactan, directly inhibits NET-associated factors (Hasler *et al.*, 2016; Lee *et al.*, 2015). Even though the mechanism and signaling cascades controlling NET induction in response to fungi remain poorly understood, the data above indicate the involvement of NET in the host response against fungal invasion.

3.4.6.1.4. Parasitic infections

Even though NETs are a potent pathogen killing mechanism, they are not omnipotent. For instance, *Leishmania infantum* was found to be resistant to NET killing thanks to an endogenous nuclease activity (Guimarães-Costa *et al.*, 2014). However, other *Leishmania* species are susceptible to NET-provided assassination. *L. donovani* inducing NETosis in a dose-dependent manner and this generation is ROS-independent. Another ROS-independent NETosis was observed in response to *L. amazonensis*. Specifically, it was demonstrated that both PAD4 and MPO inhibitors reduced NET formation induced by *L. amazonensis*. However, parasites can also induce ROS-dependent NETosis. For example, NETs induced by *Trypanosoma cruzi* were abolished by DPI inhibition (Bonne-Année *et al.*, 2014). Additionally, NET formation in response to *Plasmodium falciparum* infection has been associated with the induction of an autoimmune response that varies between children and adults. In children, circulatory NETs adherent to parasites were associated with potentially damaging antinuclear antibodies against double stranded DNA (Hasler *et al.*, 2016; Morgado *et al.*, 2015).

The exact role of NETs during parasitic host invasion remains unknown. However, results of thes studies indicate that NETs prevent parasite spreading using discrete signaling pathways.

3.4.6.2. NETosis in autoimmunity

NET structures, featuring long chromatin filaments decorated with granular proteins, gives them the potential to act as physical and antimicrobial barriers that first entrap and then kill

pathogens at the site of inflammation (Brinkmann *et al*., 2004b; Buchanan *et al*., 2006; Urban *et al.*, 2006). As described in the previous section, NETs are found in a variety of infections. However, NET formation can also occur in non-pathogenic conditions, such as autoimmune diseases (Hakkim *et al.*, 2010; Pinegin *et al.*, 2015). Prolonged formation of NETs due to impaired NET degradation is thought to be associated with autoimmune diseases such as systemic lupus erythematous, rheumatoid arthritis, psoriasis, etc. (Bonanni *et al.*, 2015; Hakkim *et al.*, 2010; Lee *et al.*, 2017). This chapter will be devoted to how NETs impact autoimmunity.

3.4.6.2.1. Systemic lupus erythematosus (SLE) and NETs

SLE is an autoimmune disorder that causes chronic inflammation in connective tissues, such as cartilage and the lining of blood vessels. The signs and symptoms of SLE involve different organs, and its development is described as multifactorial. There is no cure for SLE, and the source of the antigens targeted by the immune system remains unknown (Kaul *et al.*, 2016).

Patients with SLE have high levels of anti-ribonucleoprotein and anti-DNA antibodies in their serum, and it is speculated that these antibodies are induced by NET components (Gupta and Kaplan, 2016). Likewise, these patients have a distinct low-density granulocyte (LDG) population, which is not found in healthy controls (Denny *et al*., 2010). It was shown that LDGs have an enhanced capacity to produce NETs spontaneously and to generate large quantities of IFN-α (Lood *et al.*, 2016).

Several animal studies have reported a relationship between SLE and NETs (Banchereau and Pascual, 2006; Garcia-Romo *et al.*, 2011; Sokolove *et al.*, 2013). These studies revealed that ribonucleoprotein immune complexes induce NET formation and require mitochondrial ROS for maximal NET stimulation. In turn, extracellular release of oxidized mitochondrial DNA is proinflammatory *in vitro*, and when this DNA is injected into mice, it induces type 1 interferon synthesis and endothelial damages, indicating that mitochondrial ROS play a role in the induction of NETosis during SLE (Banchereau and Pascual, 2006; Döring *et al*., 2012a). Furthermore, the impact of SLE-targeted therapies was tested on NET formation. Celastrol, a triterpenoid compound that downregulates mitochondrial ROS (Garcia-Romo *et al.*, 2011), inhibits the neutrophil oxidative burst and NET formation induced by TNFα. Another compound was tested, called tofacitinib – a JAK inhibitor that blocks signaling downstream of multiple cytokines implicated in lupus pathogenesis. Both spontaneous and LPS-induced NETosis were significantly decreased in neutrophils obtained from tofacitinib-treated mice (Sokolove *et al*., 2013). Together, these observations support a role for NETs in the pathogenesis of SLE (Grayson and Kaplan, 2016; Lee *et al.*, 2017)

3.4.6.2.2. NETosis in psoriasis

Psoriasis is a chronic multifactorial, inflammatory skin disease that affects about 3% of the human population (Gudjonsson and Elder, 2007). Chronic inflammation during psoriasis is characterized by the intense proliferation and aberrant differentiation of keratinocytes, and the infiltration of the epidermis with lymphocytes and neutrophils. The major inflammatory molecules that characterize psoriasis are TNF-α, IFN-γ, TGF-β, and interleukins (including IL-1, IL-17, and IL-22) (Martin *et al*., 2013). Moreover, an increased number of NETotic cells

was observed in the peripheral blood of patients with psoriasis compared to healthy subjects (Hu *et al*., 2016). Several mechanisms that might explain involvement of NET formation in the disease progression were described. For example, it was shown that disrupted mechanism of reactivity to self-DNA by plasmacytoid dendritic cells (pDC) plays a role in the inflammation that activates the skin to react with hyperkeratosis in psoriasis. Normally, pDCs have no reactivity to self-DNA. However, analysis of the affected skin from psoriatic patients revealed the presence of LL-37/DNA and LL37/RNA complexes (Herster *et al*., 2019). It was shown that these complexes co-localize with DC clusters in the epidermis. Moreover, the LL-37/DNA complex colocalizes with pDC, while LL-37/RNA complex with myeloid DC (mDC). Likewise, it was revealed that peptide LL-37 colocalize with TLR9 inside the endosomes of pDCs in the affected skin (Lande *et al*., 2007; Skrzeczynska-Moncznik *et al*., 2012). To investigate the molecular mechanism of this process, insoluble LL37/DNA complexes were added to the culture of pDC. It was shown that LL37/DNA complex enters TLR9-containing endosomes and induces a powerful IFN-α response. To summarize, LL37/DNA complexes play an important role in psoriasis progression. Even though it is known that neutrophils also can produce LL37, the source of LL37 in the context of psoriasis appears to be predominantly endothelial cells of skin vessels, which produce and release LL37 in response to inflammatory cytokines (Hwang *et al*., 2014; Martinelli *et al*., 2004; Takahashi *et al*., 2010). So what is the source of DNA in psoriasis? Like it was mentioned in previous sections LL37 alone can induce NET formation. Thus, it was shown that neutrophils undergoing extracellular trap formation are the origin of DNA detected in psoriasis (Skrzeczynska-Moncznik *et al.*, 2012a).

Apart from LL37/DNA, another complex was found to colocalize with pDCs in psoriatic skin, i.e. DNA/NE/SLPI. In the affected parts of the psoriatic skin, the DNA/NE complex was found in association with secretory leukocyte proteinase inhibitor (SLPI). SLPI is expressed by neutrophils (Jacobsen *et al*., 2008; Jin *et al*., 1997; Wingens *et al*., 1998) and controls serine protease activity. SLPI is thought to play an essential role in limiting protease-mediated tissue injury associated with inflammation, especially at mucosal/epithelial surfaces, as well as in wound healing (Reardon *et al*., 2011; Zhu *et al*., 2002). The main target for SLPI is an enzyme of the neutrophil primary granules, NE, that plays a crucial role in chromatin decondensation during NETosis. *In vitro* experiments demonstrated that addition of DNA/NE/SLPI complexes into the culture of pDC induces great production of type I IFNs (Hu *et al*., 2016; Skrzeczynska-Moncznik *et al*., 2012b). Thus, all the above findings support the involvement of NETs and associated proteins in psoriasis pathogenesis.

3.4.6.2.3. NETs in Rheumatoid arthritis (RA)

Rheumatoid arthritis is the most commonly diagnosed type of autoimmune inflammatory arthritis. It affects almost 1% of the world population. Like most autoimmune diseases, RA etiology is multifactorial. Rheumatoid arthritis causes pain, swelling and, if untreated, progressive damage to joints that leads to increased morbidity and death. Inflamed joints of RA patients are massively infiltrated with neutrophils (Aletaha and Smolen, 2018). Around 80% of RA patients have autoantibodies to citrullinated protein antigens (CPAs) in their blood (Keidel *et al*., 2014). Recent research suggests that ACPAs play a crucial role in RA. A potential mechanism that leads to ACPA generation in RA was highlighted in several studies (Aletaha and Blüml, 2016; Stadt *et al*., 2011). Autoantibodies to CPAs contain nuclear

proteins (*e.g.*, histones), cytoplasmic proteins (*e.g.*, vimentin and enolase), membrane-associated proteins (*e.g.*, myelin basic protein), and extracellular proteins (*e.g.*, filaggrin, collagen II, fibrinogen, and calreticulin) (Kurowska *et al*., 2017). Anti-citrullinated-H2A/H2B-reactive RA antibodies selectively recognize NETs produced by RA joint neutrophils (Corsiero *et al*., 2016). Thus, it was suggested that NETs are a source of ACPAs implicated in RA pathogenesis. Moreover, neutrophils express and degranulate high levels of PAD2 and PAD4, leading to their accumulation in the synovial fluid (SF) of RA patients (Spengler *et al*., 2015).

In addition, NETing neutrophils were detected in the peripheral blood, synovial fluid, synovial tissues, rheumatoid nodules and skin of RA patients (Khandpur *et al*., 2013). Furthermore, the number of NETotic neutrophils in the peripheral blood of RA patients significantly correlated with the levels of ACPA in the serum (Khandpur *et al*., 2013). Additionally, it was shown that NETs can induce an inflammatory phenotype in synovial fibroblasts (SFL). NET-activated SFLs were internalizing peptides associated with NETs via RAGE-TLR9 signaling pathway promoting upregulation of MHC class II. Thus, activated SFL were presenting internalized NET-associated citrullinated peptides loaded onto MHS class II to Ag-specific T cells (Carmona-Rivera *et al*., 2017; Grayson and Kaplan, 2016). In conclusion, the results listed above reveal a credible link between RA pathogenesis and NETs (and their components).

3.4.6.2.4. NETs in vascular inflammation and thrombosis

Vasculitis is a chronic auto-inflammatory condition characterized by the presence of anti-neutrophil cytoplasm autoantibodies (ANCAs) (Kessenbrock *et al*., 2009). Thus ANCA react

with components of neutrophilic granules and monocytic lysosomes. Vasculitis associated with ANCA is classified into three distinct vasculitides, which involve inflammation of the small- and medium-sized blood vessels: microscopic polyangiitis (MPA); granulomatosis with polyangiitis (GPA, previously known as Wegener granulomatosis); and eosinophilic GPA (previously known as Churg-Strauss Syndrome) (Kallenberg, 2014). Microscopic polyangiitis and eosinophilic GPA feature autoantibodies against MPO, while granulomatosis with polyangiitis features autoantibodies against proteinase 3 (PR3). The presence of autoantibodies against NET-associated enzymes (MPO, PR3), and the finding that ANCA can induce NETosis in neutrophils, together suggest NET involvement in disease pathogenesis. In this respect, extracellular DNA traps associated with MPO and PR3 were detected in kidney biopsies from patients with active glomerulonephritis (ANCA vasculitis that affects kidneys) (Kessenbrock *et al*., 2009). Also, the number of NET products positively correlated with disease activity (Söderberg *et al*., 2015).

Moreover, ANCA-associated inflammation of blood vessels commonly leads to thrombosis, and thrombi from these patients are rich in NETs (Kambas *et al*., 2014). Finally, it was proposed that NETs might be directly associated with thrombosis development. Intravascularly formed NETs interact with fibrinogen, thus promoting fibrin deposition, and this fibrous meshwork promotes platelet activation and aggregation (Fuchs *et al*., 2010; Hasler *et al*., 2016). More work has to be done to elucidate the mechanism of NET involvement during vascular inflammation and thrombosis. However, evidence already obtained points to the existence of a link between the NET formation and disease progression.

3.4.6.2.5. NETs in atherosclerosis (AS)

Atherosclerosis (AS) is a chronic inflammatory disease. During AS, arteries harden through a build-up of plaques in the intima of blood vessels (Tuttolomondo *et al*., 2012). Main classical risk factors of AS include dyslipoproteinaemia, diabetes, smoking, hypertension, and genetic abnormalities. The inflammation is initiated by infiltration and retention of cholesterol-rich apolipoprotein B (apo B)-containing lipoproteins in the artery wall (Shapiro and Fazio, 2017). This accumulation later leads to an arterial injury that causes endothelial dysfunction, promoting infiltration of the vessel wall by neutrophils, macrophages, and DCs (Linton *et al*., 2000).

There are several lines of evidence supporting a role for NETs in the initiation and maintenance of the chronic inflammatory process in AS. It is known for example that NETs are the source of LL-37/DNA complexes (or its murine equivalent, DNA/Cramp) that are detected in atherosclerotic plaques (Döring *et al*., 2012a, 2012b). In mice fed with a fat-enriched diet, resulting in a significant deterioration of the vessel walls, the level of Cramp, co-localized predominantly with neutrophils, was dramatically increased in the aortic walls. Likewise, feeding Cramp-/- mice the same fat-enriched diet caused significantly less damage to blood vessels in comparison to wild-type mice. There were also considerably fewer neutrophils in the aorta walls of Cramp-/- mice compared to control animals. In humans, more NET markers (i.e., complexes of citrullinated histones or MPO with DNA, nucleosomes), were found in tissue sections of coronary vessel walls of 282 patients with coronary artery diseases (Borissoff *et al*., 2013). Finally, in a mouse model of AS in which apolipoprotein E (ApoE)-deficient mice were fed a high-fat diet for eight weeks, NET accumulation surrounded

cholesterol crystals in atherosclerotic lesions (Yamamoto *et al*., 2018). To test NET involvement in this AS disease model, two neutrophil proteases, NE (also known as ELANE) and PR3, were deleted in the mice lacking ApoE. The resulting triple knockout animals had less NETosis rate, as well as smaller atherosclerotic lesions and lower systemic IL-1β levels, compared to ApoE-/- mice expressing NE and PR3 (Jorch and Kubes, 2017; Warnatsch *et al*., 2015). This again suggests a potential role for NETs and/or these proteases in AS.

This chapter was dedicated to the role of NETs during various pathophysiological conditions. In summary, the relevance of NETs seems clear but the impact is controversial. NET generation is an important mechanism of host defence against bacterial and parasitic invasion, as well as against fungi and viruses. However, constitutive NET formation during chronic inflammation of various origins contributes to disease progression. Further studies are needed to understand the singling pathways that control NET formation in response to physiological stimuli. Thus, understanding of NET formation mechanisms associated with specific diseases may lead to the identification of important therapeutic targets.

3.4.7. Methods for the detection of NETs

The initial description of NETs as an antimicrobial response, as well as their involvement in various diseases, generated widespread interest to study the underlying mechanisms, as well as the upstream signaling pathways. For this purpose, several approaches have been used that are described in this section.

3.4.7.1. Electron microscopy

Brinkmann *et al*. used electron and fluorescence microscopy for NET visualization. They confirmed that NETs consist of chromatin that is associated with granular and nuclear proteins (such as elastase, cathepsin G, MPO; and the histones H1, H2A, H2B, and H3, respectively). Their study, therefore, established NET "markers" that were used by other researchers afterward (Brinkmann *et al*., 2004b). However, electron microscopy is not a very practical approach to quantify NETs in large amounts of samples.

3.4.7.2. Fluorescence microscopy

Chromatin being at the core of NET structure, every immunohistochemistry protocol of NET visualization includes DNA labeling. However, even if there is an argument that extruded decondensed chromatin accumulates more signal than the packed chromatin remaining in the cell, the use of a DNA dye alone is not sufficient for NET quantification. Thus, additional markers are generally used along with DNA labeling with MPO and NE being the most common ones (Carmona-Rivera and Kaplan, 2016; Mitroulis *et al*., 2011a; Papayannopoulos *et al*., 2010b). However, this approach suffers from a potentially significant drawback. Given the propensity of several neutrophil granule proteins to strongly associate with cell membranes upon their release from the cells (Owen *et al*., 1995; Pryzwansky *et al*., 1979), the above observation raises the possibility of an abundant nonspecific signal. A crucial control is lacking, i.e., the digestion of NETs with DNase, to determine if all of the

fluorescence is in fact associated with NETs (as total fluorescence is typically counted by investigators using this approach).

3.4.7.3. Spectrofluorometric quantification of NETs

Spectrofluorometric NET quantification is widely used to investigate NETosis. It is based on the staining of DNA released during NET formation, using a DNA dye (such as Sytox green) described as being non-permeable dye by its manufacturer. Thus, extruded DNA is quantified and standardized to total DNA (using another DNA dye that is cell permeable, such as DAPI). This method is straightforward and allows to analyze numerous samples at once. However, it can't be considered as reliable, as its correlation with microscopy observation is unsatisfactory. NET morphology can vary depending on the stimulus (yielding either spread or diffused NETs); also, different inhibitors might affect different steps of NET formation (decondensation or extrusion), and none of these factors can be verified without microscopy. Likewise, dead and lysed cells are typically counted as NETing cells, contributing to a strong false positive signal. Furthermore, Sytox Green should be used at concentrations of 500 nM or less in eukaryotic cells according to its manufacturer, whereas most investigators use it at 3 µM or more (Gray *et al*., 2013; Gupta *et al*., 2014; Khan *et al*., 2017; White *et al*., 2016). There is therefore a definite risk of a nonspecific signal that should be accounted for, but usually it is not.

Thus, methodological approaches of NET quantification listed above suffer from significant inherent drawbacks. The biggest issue is the inclusion of an abundant nonspecific signal. This crucial issue will be fully addressed in this thesis.

4. Chronic inflammation

As discussed earlier, physiological inflammation is a pivotal part of the innate immune system that is self-regulated through various molecular feedback loops which enables the resolution of the process, withdrawal of the infection cause and restoring the tissue to a regular state. However, the process is occasionally unable to terminate, prolonging for months, or even years. This state is referred to as chronic inflammation, and is recognized as a cause or comorbidity of many contemporary diseases (Pahwa and Jialal, 2019).

The causes of chronic inflammation are not entirely elucidated, but there seem to be a few common elements. The onset of acute and chronic inflammation appears to be the same. However, the process advances into a chronic condition either because the immune system fails to eradicate the infection cause, and the source persists; or the course of the process is unable to terminate even if the initial cause has been eliminated. In a smaller number of cases, a low level of ungoverned inflammatory response persists over time, with no apparent reason. Some autoimmune disorders, like systemic lupus erythematosus or rheumatoid arthritis, also carry a chronic inflammatory component, as the innate immune system mistakenly attacks host antigens (Garn *et al*., 2016; Pahwa and Jialal, 2019).

Available data reveal several predisposing risk factors that increase the probability of developing a disease associated with chronic inflammation. Unadvisable nutritional habits accompanying our contemporary lifestyle are among the forerunners of a myriad of diseases bearing a chronic inflammation component (Minihane *et al*., 2015). Low-quality, industrial food with high-calorie content that is typically consumed in developed countries, induces the accumulation of fatty tissue, secreting adipokines and other pro-inflammatory cytokines. Smoking is another behaviorally modifiable risk factor, associated with prolonged inflammation (Lee *et al*., 2012). Everyday stress, another hallmark of modern lifestyle, accompanied by irregular sleep patterns, elevates cytokine production and represents another probable cause (Liu *et al*., 2017). Advanced age and misbalanced hormonal status have been repeatedly correlated with chronic inflammatory diseases (Okin and Medzhitov, 2012). In sum, poor dietary habits, smoking, sedentary lifestyle accompanied by an aging population, all symbols of present-day lifestyle in developed countries, represent potent risk factors for developing chronic inflammation-related diseases.

Chronic diseases are long term, slowly progressing disorders demanding constant medical attention and ultimately reducing the quality of life. Altogether, these disorders represent a significant health burden in the western world as it is estimated that they indirectly cause roughly 60% of deaths globally every year (Morabia and Abel, 2006). Some chronic diseases with a strong autoimmune component like atherosclerosis and diabetes, are followed by aftermaths such as myocardial infarction and stroke. Several types of common carcinomas including lung, ovarian, prostate and pancreatic cancers also develop a persistent immune response. In all, chronic inflammation can affect almost every tissue, provoking various

diseases, like chronic obstructive pulmonary disease, rheumatoid arthritis, Alzheimer's disease, allergic asthma, chronic kidney disease, and inflammatory bowel disease.

Some commonly used medications such as aspirin were found to relieve the symptoms of chronic inflammation, but often corticosteroids are prescribed or non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen (Straub and Cutolo, 2016).

It is generally accepted that effector cells dominating the chronic inflammatory response are macrophages and T cells as opposed to acute inflammation where the principal inflammatory cells are neutrophils (Miyasaka *et.al.*,2016). The role of neutrophils in chronic inflammation is therefore less elucidated. For long it has been thought that their main contribution to chronic illness is through damaging host bystander cells, as they exhibit relatively low target specificity. However, new emerging evidence brings together a broader picture (Soehnlein *et al*., 2017).

Chronic inflammation causes local changes in the inflamed tissue like fibrosis and a remodeled vascular network (Kis *et al*., 2011), but as time progresses systemic metabolic changes may occur. Hyperglycemia and hyperlipidemia accompanying the advancement of pathogenesis provoke extramedullary production of neutrophils, augmenting the influx to circulation (Nagareddy *et al*., 2013).

Neutrophils have been implicated in the onset and progression of type-1 diabetes (Huang *et al*., 2016a). The granular cargo released during the course of inflammation was shown to

impair islet cells. This initiates the diabetogenic T-cell response and further advancement of disease. Anti-neutrophil treatments were demonstrated to delay and restrict the progression of type-1 diabetes (Huang *et al*., 2016b).

Chronic obstructive pulmonary disease (COPD) is a wide-spread condition characterized by progressive tissue deterioration. A large body of evidence suggests that proteases released by neutrophils such as elastases are among the mediators of COPD (Hoenderdos and Condliffe, 2013).

The role of neutrophils in allergic asthma is also backed by clinical evidence. Neutrophils are recruited into lungs of affected patients, especially during the acute phase of disease, promoting and prolonging symptoms. Also, neutrophil-attracting chemokines like IL-8 and IL-17 are prominent performers in uncontrolled asthma (Gao *et al*., 2017). Several studies showed that neutrophils also have a role in sensitization to different allergens like pollen. Exposure to allergens leads to neutrophil accumulation, which later on facilitates sensitization through insufficiently understood mechanisms (Arebro *et al*., 2017).

Chronic kidney disease is another condition affected by inadequate neutrophil activity. Reactive oxygen species have been implicated in the development of proteinuria, modification of glomerular filtration pace and overall change in morphological and functional properties of glomerular cells (Stock *et al*., 2018). Matrix metalloproteinases and leukotrienes released by granular discharge cause changes in tissue structure and vascular network of the kidney.

Further on, the overexpression of adhesion molecules accompanying neutrophil migration was associated with the severity of glomerulonephritis (Mayadas *et al*., 2010).

Chronic neurodegenerative disease, like Alzheimer's is also influenced by neutrophils. Evidence suggests that granular proteins like neutrophil elastases and cathepsins exert neurotoxic effects (Stock *et al*., 2018). A recent study demonstrated that blocking a neutrophil receptor in a mouse model averted the influx of neutrophils to the brain, diminishing amyloid plaque and microgliosis. These animals also showed improvement in long-term memory (Cruz Hernández *et al*., 2019).

Another inflammatory condition where neutrophils play an essential role is chronic gout (So, 2013). Because this particular context is central to the work done in this thesis, it will be examined in more detail in the next section.

4.1. Gouty arthritis

Gout or gouty arthritis is the most common cause of chronic inflammatory arthritis; over 2% of people diagnosed with rheumatoid arthritis will also develop gout (Jebakumar *et al*., 2013), and the number of patients that have both has steadily increased in past years. Prevalence of gout in men is much higher than in women, and the burden of disease increases with a rise of the underlying risk factors (Roddy and Choi, 2014; Roddy *et al*., 2007). Several lifestyle-dependent factors such as alcohol consumption, meat consumption, and a high BMI increase the risk of gout (Lee *et al*., 2006).

The prerequisite of gout is the hyperuricemia, a condition so defined when serum uric acid levels rise above 6.8 mg/dl – the concentration of urate solubility at physiologic temperature and pH. When uric acid concentration exceeds the limit of solubility, it crystallizes as a sodium-containing salt (Neogi, 2012). Monosodium urate crystals (MSU) cause an extremely acute inflammatory reaction, that is commonly associated with a massive infiltration of neutrophils (Ryckman *et al*., 2003). Clinically, gout is described as repeated episodes of acute arthritis involving one joint at a time. The most common clinical characteristics of gout are its sudden onset, which is associated with a high level of pain and swelling of the affected joint that reaches its peak within approximately 6 to 12 h. After few days gouty arthritis undergoes spontaneous resolution (Eggebeen, 2007; Pittman and Bross, 1999; Steiger and Harper, 2014).

The exact mechanisms of gout initiation and resolution are not clearly defined although there is a model that can be suggested, based on the data available (Figure 7). Experiments with human serum *in vitro* demonstrated that MSU can activate a broad range of complement proteins of both the classical and alternative pathways. The activation of many complement proteins appears to involve direct interactions with the negatively-charged crystal surface, resulting in the generation of active C5a and C5b (Martin and Harper, 2010; Pekin and Zvaifler, 1964; Popa-Nita and Naccache, 2010; Tramontini *et al*., 2004). Thus, complement complexes associated with MSU induce activation of residential macrophages, which in turn produce pro-inflammatory cytokines (IL-1 beta, IL-8, and TNF alpha). Pro-inflammatory cytokines like IL-1 beta increase surface expression of adhesion molecules on endothelial cells. Both IL-8 produced by macrophages and up-regulation of E-selectin expression by endothelial cells can induce neutrophil recruitment to the site of MSU deposition. The

consequences of neutrophil interaction with MSU crystals include the synthesis and release of a large variety of mediators that include ROS (*e.g.* O_2^-, H_2O_2, O_2, NO), LTB4, PGE2, anti-microbial peptides, enzymes, cytokines, chemokines such as IL-8, and S100A8 and S100A9 (Popa-Nita and Naccache, 2010; Ryckman *et al*., 2003; Simchowitz *et al*., 1982). Neutrophils stimulated with MSU also show a significant delay in apoptosis, presumably leading to their persistent and prolonged activity during gouty inflammation (Akahoshi *et al*., 1997). Both increased recruitment and prolonged lifespan likely work together to accumulate large numbers of neutrophils within the joint.

As for the resolution of gout, several factors have been described to contribute to the process. It was shown that synoviocytes exposed to complement complexes isolated from arthritic joints produced the monocyte chemoattractant, MCP-1/CCL2 (Martin and Harper, 2010). Recruited during the initiation phase of inflammation, monocytes differentiate into anti-inflammatory macrophages that start to produce TGFβ (Yagnik *et al*., 2004). Another likely mechanism that triggers resolution in gout is the clearance of apoptotic leukocytes by macrophages. In acute inflammation, the recognition and ensuing phagocytosis of apoptotic neutrophils by macrophages also induces a switch from the activated inflammatory phenotype to one that promotes resolution and involves TGFβ secretion (Fadok *et al*., 1998). Some of the molecules decorating MSU crystals are also potentially involved in gout attack resolution. Analysis of such proteins from MSU crystals isolated from both gout patients and animal models during the early stages of inflammation, showed that crystals are initially coated with complement-activating IgG. However, during the resolution phase, IgG is displaced by apolipoproteins (Martin and Harper, 2010; Ortiz-Bravo *et al*., 1993). The deliberate coating

of MSU crystals with apolipoproteins can indeed suppress ongoing activation of complement proteins (Ortiz-Bravo *et al*., 1993). An additional factor that is potentially involved in the resolution of MSU-induced inflammation was described recently. It was shown that MSU-activated neutrophils form NETs that aggregate around the crystals. These aggregated NETs, like NETs induced by other stimuli, feature neutrophil proteases that can degrade inflammatory mediators (cytokines and chemokines) produced by neutrophils in response to MSU, thereby promoting the resolution of MSU-induced inflammation (Figure 7) (Schauer *et al*., 2014).

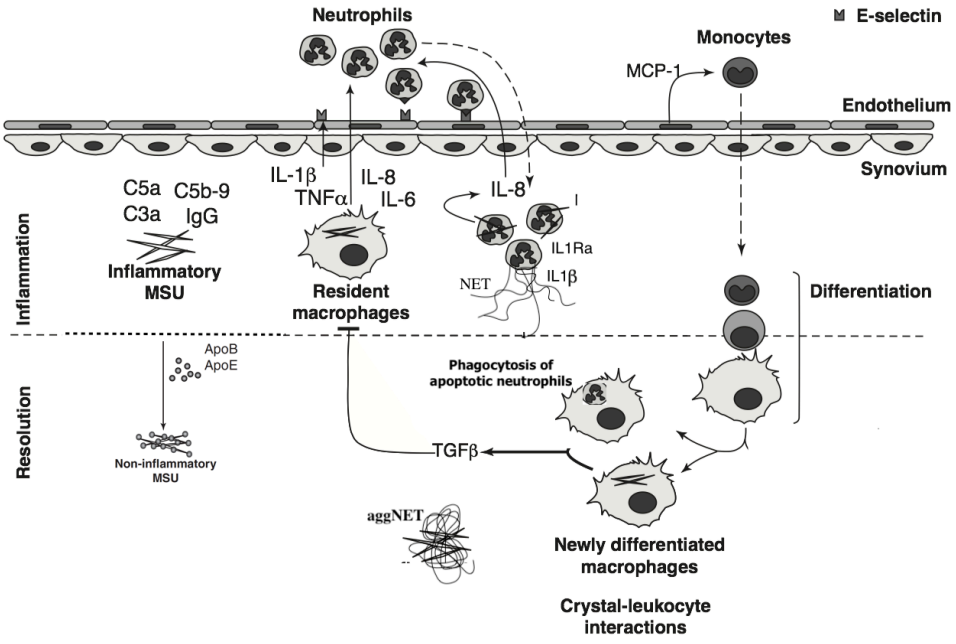

Figure 7. Proposed model of monosodium urate (MSU) crystal-induced inflammation.

Complement proteins are cleaved and activated at the crystal surface, whereas tissue-associated macrophages phagocytose MSU crystals and generate proinflammatory cytokines. These signals initiate and augment neutrophil recruitment, aided by the activation of adhesion molecules such as E-selectin on endothelial cells. Recruited neutrophils activated after contact with MSU crystals, produce NETs and interleukin (IL)-8, IL1β, etc. augmenting neutrophil accumulation. Differentiation of recruited monocytes into macrophages allows TGFβ production after either MSU stimulation or uptake of apoptotic neutrophils and contributes to resolution. Simultaneously, coating of MSU crystals with apoliprotein (Apo) B and Apo E blocks ongoing activation of complement proteins and local cells. Aggregated NETs

(aggNET) contribute to the resolution by degrading inflammatory mediators. (adapted from Martin and Harper, 2010 , authorization requested ref # 4657311356754)

Drugs currently used for the treatment of the acute gout attack inhibit the amplification of the inflammatory response to MSU crystals. For example, colchicine, a drug with clinical efficacy in acute gout (Ahern *et al*., 1987), inhibits neutrophil recruitment and activation (Nuki, 2008; Roberge *et al*., 1994). Non-steroidal anti-inflammatory agents (NSAIDs) prevent release of PGE_2 and other AA metabolites from various cells in response to MSU crystals (Gordon *et al*., 1985; Pouliot *et al*., 1998). However, these treatments also lead to undesirable side-effects, and not all patients respond satisfactorily (Information *et al*., 2018). There is therefore a need to learn more about MSU signaling, in order to target the various neutrophil products made in response to MSU. This is a prerequisite to the development of novel treatments to prevent or treat acute gout attacks, and will be explored in this thesis.

4.1.1. MSU signaling

The receptor (or complex of receptors) specifically recognizing MSU crystals at the surface of human neutrophils is still unidentified. Various proteins can be absorbed on the surface of MSU crystals, each of which could potentially bind to a surface receptor on neutrophils. It was reported that MSU activates both the classical and the alternative complement pathways *in vitro* (Doherty *et al*., 1983; Hasselbacher, 1979), and that complement components (*e.g.* C1q, C1r, and C1s) can all bind to MSU crystals. Additionally, MSU crystals avidly bind to IgG; however, activation of the classical complement pathway by MSU does not require the presence of immunoglobulins (Giclas *et al*., 1979).

Recent studies of the mechanisms that drive neutrophil activation by MSU crystals have led to the suggestion that Src family tyrosine kinases (Popa-Nita *et al*., 2007), PKC (Popa-Nita *et al*., 2009), and PI3Ks (Popa-Nita *et al*., 2007) are key signaling events. Thus MSU crystals activate classical PKC isoforms, and this activation is necessary for the MSU-induced degranulation and generation of chemotactic activity in neutrophil supernatants (Popa-Nita *et al*., 2009). Evidence was also obtained that the tyrosine kinase, Syk, is a substrate of PKC and that the PKC-mediated serine phosphorylation of Syk is necessary for its interaction with the regulatory subunit of PI3K (p85), and thus to the subsequent activation of PI3Ks, as well as that of the PI3K effector, Akt (Popa-Nita *et al*., 2007). However, MSU crystals induced the formation of a complex containing p85 and Syk, which was entirely abrogated by PP2, a Src family kinase inhibitor, suggesting that Src-mediated tyrosine phosphorylation of Syk is essential for its interaction with p85 (Popa-Nita *et al*., 2009). Likewise, it was shown that MSU crystals induce the rapid tyrosine phosphorylation of Tec in a Src-dependent manner (Popa-Nita *et al*., 2008). Inhibition of Tec reduced IL-8 production induced by MSU, which indicates that Tec is necessary for the MSU-induced secretion of IL-8 from human neutrophils (Popa-Nita *et al*., 2008). In addition to IL-8, MSU is also known to promote the release of IL-1β and IL-1ra from neutrophils (Torres *et al*., 2009). The observation that MSU can activate PI3K (Popa-Nita *et al*., 2007), along with a recent demonstration that this kinase is involved in cytokine production (including that of IL-8) (Fortin *et al*., 2011), suggests that PI3Ks might contribute to MSU-induced cytokine production.

In addition to cytokine and lipid mediator production, degranulation, and oxidative burst, MSU-activated neutrophils have also been shown to form NETs (Schorn *et al*., 2012). However, pathways that control NET formation in response to MSU are poorly understood. Metroulis *et al.* demonstrated that both serum and synovial fluid from gout patients can induce NET formation by neutrophils from healthy donors. An IL-β inhibitor (Anakinra) partially prevented this response, showing that NET formation during gout might be driven at least in part by IL-1β. Additionally, NET formation in response to MSU was abolished by 3-methyladenine (3-MA), which is commonly considered as an autophagy inhibitor, although its primary target is class III PI3K (Mitroulis *et al*., 2011). More evidence for a role of PI3K was obtained by use of the pan-PI3K inhibitor, LY294002, which also significantly prevented NET formation in response to MSU. In contrast, inhibition of Src had no effect. (Mitroulis *et al*., 2011). Weather NADPH oxidase is required for MSU-evoked NET release remains controversial. Schorn *et al*. demonstrated that in the presence of anti-oxidants (BHT, BHA and ascorbic acid) MSU-induced NET formation was disrupted, although no quantification data was presented (Schorn *et al*., 2012). Another study claimed that MSU-activated neutrophils from CGD patients failed to form aggregated NETs, also without relying on quantitative data analysis (Schauer *et al*., 2014). However, quantitative analysis performed by Linden *et.al* showed no significant NET inhibition by DPI in response to MSU (Linden *et al*., 2017). The same study elucidated effect of p38 MAP kinase, Syk, mTORC2 and PI3K inhibition on MSU-induced NETosis. Inhibition of PI3K using wortmannin showed no effect, while inhibition of Syk, p38 and mTORC2 significantly decreased the NETosis rate (Linden *et al*., 2017).

Collectively, the molecular mechanisms underlying MSU-induced responses are not fully understood. There have been studies providing insights into the role of various kinases, but these studies on MSU-elicited NETosis have yielded controversial results. Further research into MSU signaling are therefore needed, and will be investigated in this thesis.

HYPOTHESES AND OBJECTIVES

The last decades of research provided new insights into the multiple roles of neutrophils during both acute and chronic inflammation. It is now well recognized that neutrophils are much more than the suicidal bacterial killers previously depicted in immunology textbooks. Neutrophils actually contribute to the course of inflammation by performing various functional responses. However, the mechanisms that control neutrophil functional responses are incompletely understood. In particular, there are areas of neutrophil biology that beg to be revisited and explored.

One such area is NET formation, which is widely considered to be a ROS-dependent process despite the fact that several physiological inducers have little or no ability to activate the NADPH oxidase. Likewise, information concerning the signaling pathways upstream of NET formation is so fragmentary that few conclusions can be drawn. We therefore hypothesized that NET formation can occur independently of endogenous ROS, and that there must be some common elements among the pathways governing this response.

Our objectives (Article 1) were to:

1. Develop a specific and reliable method for NET quantification.
2. Identify signaling events involved in NET formation. in response to physiological stimuli.
3. Determine to which extent endogenous ROS are necessary for NET formation, and whether there exist other mechanims that are perhaps more central to the phenomenon.

Another area of neutrophil biology that features unresolved issues is that of MSU interactions with these cells. There is the intriguing issue of neutrophils being the first cells

to invade gouty articulations, followed by monocytes. Under several inflammatory settings, neutrophils contribute to this sequential leukocyte recruitment, but whether such a scenario applies in MSU-activated cells remains surprisingly undocumented. Likewise, the full array of proteic mediators and intermediates being generated by neutrophils in response to MSU have not been explored. Finally, the signaling pathways mobilized by MSU and contributing to its various cellular responses have only been partially investigated. In particular, the fact that some immediate-early genes induced by MSU (*e.g.* IL-1, CXCL8) normally requires transcription factor activation in neutrophils, suggests that at least some can be mobilized by MSU. We therefore hypothesized that new, biologically relevant information can be gathered by examining the genomic and proteomic changes triggered by MSU in neutrophils; and that transcription factors are involved in the onset of gene transcription in response to the crystals.

Our objectives (Article 2) were to:

1. Investigate the genomic and proteomic changes elicited by MSU interactions with neutrophils.
2. Characterize the signaling pathways mobilized in neutrophils by MSU, their interrelationships, and their impact on functional responses such as cytokine generation and NET formation
3. Characterize transcriptional events participating in cytokine gene induction by MSU

ARTICLE 1

Physiological stimuli induce PAD4-dependent, ROS-independent NETosis, with early and late events controlled by discrete signaling pathways

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Authorship

Contribution: OT carried out all experiments, compiled all the data, and wrote the first draft; PPMcD designed the research, mentored the other author, and wrote the final version of the paper.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Abstract

On connaît aux neutrophiles la capacité d'extruder leur chromatine décondensée pour former des « NETs » (*Neutrophil Extracellular Traps*). Ces structures immobilisent les agents pathogènes, ce qui empêche leur propagation et favorise leur élimination. Elles contiennent également des molécules antimicrobiennes. Les NETs influencent la pathogénèse de maladies inflammatoires chroniques, de maladies auto-immunes et de certains cancers. Malgré l'importance des NETs, les mécanismes moléculaires sous-tendant leur formation ainsi que les voies de signalisation en amont ne sont que partiellement élucidés. De même, les approches méthodologiques actuelles pour quantifier les NETs comportent d'importantes lacunes, en particulier des faux positifs parfois prédominants.

Dans cette étude, de nouveaux polymères fluorescents ne liant que la chromatine extrudée ont été utilisés, ce qui permet une quantification spécifique et normalisée de la NETose. Cela nous a permis de classer de manière fiable l'efficacité relative de divers inducteurs physiologiques des NETs. Chez les neutrophiles activés par de tels stimuli, l'inhibition des voies de signalisation Syk ou PI3K bloque la NETose en affectant sur les événements tardifs de cette réponse. L'inhibition des voies TAK1, MAPK p38 ou MEK diminue également la NETose, mais en agissant sur des événements précoces. Par contre, l'inhibition de la PKC, des kinases de la famille Src ou de JNK n'affecte en rien la NETose; la cycloheximide ou l'actinomycine D sont tout aussi inefficaces. Tel qu'attendu, la formation de NETs est profondément compromise par l'inhibition de la NADPH oxydase chez des neutrophiles activés avec le PMA, mais s'est avérée indépendante des ROS en réponse à des agonistes physiologiques. A l'inverse, nous montrons pour la première fois que l'inhibition sélective de PAD4 atténue efficacement la NETose en réponse à tous les stimuli testés. Nos données avancent considérablement les connaissances actuelles sur les voies de signalisation contrôlant la NETose, et révèlent notamment comment elles affectent les stades précoces ou tardifs du phénomène. Compte tenu de l'implication des NETs dans plusieurs pathologies, nos résultats identifier des cibles moléculaires pouvant être exploitées pour des thérapies futures.

Abstract

Neutrophils are known to extrude decondensed chromatin, thus forming NETs (neutrophil extracellular traps). These structures immobilize pathogens, thereby preventing

their spreading, and are also adorned with antimicrobial molecules. NETs can also influence pathogenesis in chronic inflammation, autoimmunity, and cancer. Despite the importance of NETs, the molecular mechanisms underlying their formation, as well as the upstream signaling pathways involved, are only partially understood. Likewise, current methodological approaches to quantify NETs suffer from significant drawbacks, not the least being the inclusion of a significant nonspecific signal.

In this study, we used novel, fluorescent polymers that only bind extruded chromatin, allowing a specific and standardized quantification of NETosis. This allowed us to reliably rank the relative potency of various physiologic NET inducers. In neutrophils activated with such stimuli, inhibition of the Syk or PI3K pathways blocked NETosis by acting upon late events in NET formation. Inhibition of the TAK1, p38 MAPK, or MEK pathways also hindered NETosis, but by acting on early events. By contrast, inhibiting PKC, Src family kinases, or JNK failed to prevent NETosis; cycloheximide or actinomycin D were also ineffective. Expectedly, NET formation was deeply compromised following inhibition of the NADPH oxidase in PMA-activated neutrophils, but was found to be ROS-independent in response to physiological agonists. Conversely, we show for the first time in human neutrophils that selective inhibition of PAD4 potently prevents NETosis by all stimuli tested. Our data substantially extends current knowledge of the signaling pathways controlling NETosis, and reveals how they affect early or late stages of the phenomenon. In view of the involvement of NETs in several pathologies, our findings also identify molecular targets that could be exploited for therapeutic intervention.

Introduction

Neutrophils are a cornerstone of the innate immune system, by virtue of their phagocytic and microbicidal activities, which greatly contribute to pathogen clearance. In this context, an important neutrophil response is their ability to extrude decondensed chromatin, thus forming extracellular structures termed NETs (for neutrophil extracellular traps)[1]. The chromatin backbone of NETs entraps various microorganisms (bacteria, viruses, yeasts, and even some parasites)[1-3], and while DNA itself can exert antimicrobial effects[4], NETs feature histones, proteases and other components, which all participate in microorganism killing. The ability of neutrophils to undergo NETosis is conserved across vertebrates, from zebrafish to mammals, and has been observed in several *in vivo* settings, suggesting that it is an important defense mechanism. Experimental evidence supports this notion, insofar as intravenous injection of DNase in animals infected with bacteria or viruses increases bacteremia or viremia[5,6], confirming that NETs act (at the very least) to prevent microorganism dissemination.

Despite the foremost role NETosis in neutrophil biology, host defence, and pathophysiology, the underlying molecular mechanisms remain only partially understood. Several studies have shown that endogenous reactive oxygen species (ROS) are needed for NET formation. Accordingly, some ROS (*e.g.* singlet oxygen, HOCl, H_2O_2) can directly induce NETs in neutrophils[7-10]. More direct evidence is that inhibiting either NADPH oxidase or myeloperoxidase prevents NET formation in response to PMA or bacteria[7,9-11]. Similarly, neutrophils from chronic granulomatous disease patients, which are unable to generate ROS[12], fail to undergo NETosis in response to PMA[7]. As a result, it has become

widely accepted that NETosis is a ROS-dependent process. This is consistent with the fact that most of the studies on NETosis have employed PMA, a powerful NADPH oxidase activator. However, the phenomenon is also known to occur in response to stimuli that are ineffective ROS inducers, such as calcium ionophores, GM-CSF, TNFα, or IL-1β[11,13], which begs for the issue to be revisited.

Arginine deimination has emerged as another potential underpinning of NETosis, insofar as citrullinated proteins, PAD2, and PAD4 associate with NETs in response to inflammatory stimuli in humans[14,15]. In addition, pretreatment of human neutrophils with the general PAD inhibitor, chloraminidine, was found to hinder NETosis[16-21]. However, the actual PAD isoform responsible for this effect has yet to be identified in human neutrophils, even though studies conducted in knockout animals have suggested PAD4 as the main citrullinating enzyme(Li *et al.*; Martinod *et al.*; Kolaczkowska *et al.*). The recent availability of a selective PAD4 inhibitor, GSK484[22], at last offers an opportunity to further explore the matter in human neutrophils.

The intracellular signaling pathways acting upstream of NETosis have also begun to be elucidated. However, the overall picture remains blurred, as it mostly consists of isolated observations concerning individual pathways, made using different stimuli, and using different methods. Thus, the Syk and PI3K pathways appear to be crucial in neutrophils stimulated by PMA, inflammatory crystals, or β-glucan[23-25,13,26,27], but Syk seems to be dispensable for NETosis triggered by FcγRIIIb clustering[28]. For p38 MAPK, Behnen *et al.* reported that it is needed for NET formation induced by immobilized

immune complexes(Behnen *et al*.), but other investigators found no involvement using different stimulatory conditions[29,30]. Similarly, MEK was reported to control NETosis in response to FcR engagement or calcium pyrophosphate crystals[23-25,13,26-28] but little is known about soluble stimuli. In the case of PKC, it was reported to be necessary for NETosis elicited by PMA or oxidized LDL[31,28,32], but not in response to mercury-containing compounds[30]. Finally, one group reported that JNK is required for NETosis in cells stimulated by PMA, LPS, or bacteria (Khan *et al*) while another group showed that TAK1 can control NET formation in response to FcRIIIB clustering[23-25,13,26,27]. In summary, much remains to be done to sort, complete, and integrate the available information.

Finally, current methodological approaches to quantify NETs suffer from significant drawbacks, in particular the inclusion of an abundant nonspecific signal. Here, we describe a NET quantification approach based on novel fluorescent polymers that only bind extruded chromatin. This allows for a specific, reliable, standardized quantification of NETosis, and was applied to decipher some of the underlying mechanisms, as well as the upstream signaling pathways controlling the phenomenon.

Materials and Methods

Antibodies and reagents. Antibodies against myeloperoxidase (A0398) were from Dako/Agilent (Mississauga, ON, Canada); antibodies against citrullinated histone H3 were from Abcam (Ab5103); phospho antibodies were from Cell Signaling (Beverly, MA, USA). Ficoll-Paque Plus was from GE Biosciences (Baie d'Urfé, Qc, Canada); endotoxin-free (<

2 pg/ml) RPMI 1640 was from Wisent (St-Bruno, Qc, Canada). Recombinant human cytokines were from R&D Systems (Minneapolis, MN, USA). Actinomycin D, cycloheximide, N-formyl-methionyl-phenylalanine (fMLP), and phenylmethanesulphonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA). Kinase inhibitors and fluorescent probes were all purchased through Cedarlane Labs (Missisauga, Canada). PlaNET reagents, fluorescent chromatin-binding polymers, were from Sunshine Antibodies (https://sunshineantibodies.com/planet-001.html).

Cell isolation and culture. Neutrophils were isolated from the peripheral blood of healthy donors, under a protocol approved by an institutional ethics committee (Comité d'éthique de la recherche du CIUSS de l'Estrie-CHUS). All subjects gave written informed consent in accordance with the Declaration of Helsinki. Briefly, whole blood was collected using an anticoagulant (sodium citrate), and successively submitted to dextran sedimentation, Ficoll separation, and water lysis – as previously described[33]. The entire procedure was carried out at room temperature and under endotoxin-free conditions. As determined by Wright staining and FACS analysis, final neutrophil suspensions contained fewer than 0.1% monocytes or lymphocytes; neutrophil viability exceeded 98% after 4 h in culture, as determined by trypan blue exclusion and by Annexin V/propidium iodide FACS analysis.

NETosis assays. For each condition, a 500-µl drop of a neutrophil suspension ($2x10^6$/ml in RPMI 1640/2% autologous serum) was deposited onto coverslips that were freshly coated with poly-L-lysine, and the cells were left to adhere for 60 min in a cell culture incubator. Inhibitors and/or stimuli were then added and the final volume brought to 550 µl,

prior to a 4-h incubation (37°C, 5% CO_2). Reactions were stopped by adding 500 µl ice-cold PBS containing 1 mM PMSF, and the coverslips were placed on ice for 10 min. At this point, one of two procedures were followed.

When antibodies were used for NET detection, the liquid on the coverslips was discarded and cells were fixed for 15 min in ice-cold PBS containing 2% paraformaldehyde, as well as a nuclear stain (*e.g.* DAPI, Hoechst 33342). The fixed cells were then washed with ice-cold PBS, and blocked for 60 min with PBS containing 5% normal goat serum (i.e. serum from the same species in which the 2nd antibody was raised), hereafter referred to as Blocking Buffer. Cells were next incubated in PBS containing the primary antibody (anti MPO, 1:1000) for 90 min, washed, and incubated 45 min with an Alexa 568-labeled secondary Ab (goat anti-rabbit, Molecular Probes #A11011, 1:2000) in Blocking Buffer. Coverslips were then mounted onto glass slides using a drop of mounting medium (ProLong Gold, Life Technologies) and sealed, prior to epifluorescence microscopy analysis.

When PlaNET reagents were used for NET detection, the liquid on the coverslips was discarded and cells were incubated (90 min on ice, with gentle shaking) in 1 ml of PBS containing 1 mM PMSF and diluted PlaNET reagent (as recommended by the manufacturer). Cells were finally fixed (15 min, room temperature) in PBS containing 2% paraformaldehyde, as well as a nuclear stain. The fixed cells were washed once with PBS, and the coverslips were mounted as described above, and analyzed by epifluorescence microscopy. For quantitation, 3 fields at 10x magnification were counted, that never included the coverslip edges; this amounts to about 1,000 neutrophils in total.

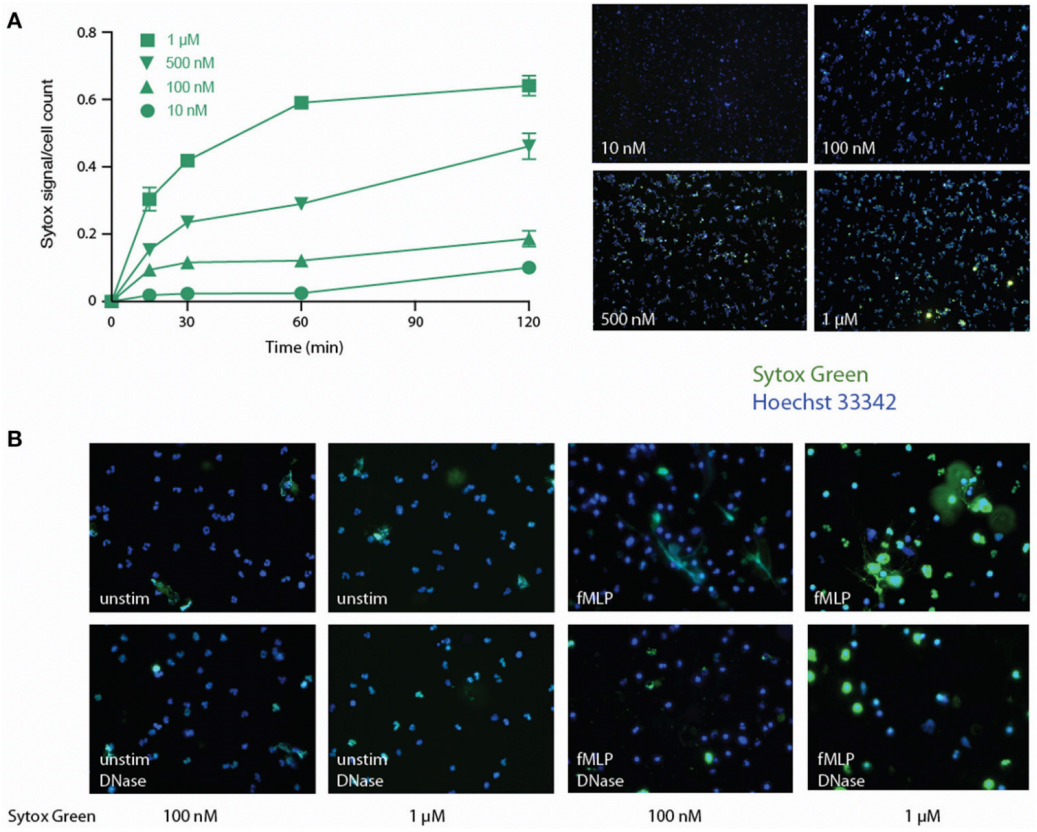
Immunoblots. Samples were prepared, electrophoresed, transferred onto nitrocellulose, and processed for immunoblot analysis as previously described[34,35].

Data analysis. All data are represented as mean ± SEM. Unless otherwise stated, statistical differences were analyzed by Student's t-test for paired data, using Prism 7 software (GraphPad, San Diego, CA, USA).

Results

A new approach to visualize and quantify NET formation. Various procedures have been used for this purpose, yet they all suffer significant drawbacks, not the least of which is the inclusion of a false positive fluorescence signal. A widespread approach is to incubate neutrophils with a DNA dye (*e.g.* Sytox Green) that is described as cell-impermeable by its manufacturer, and to analyze total fluorescence in the supernatants. However, we observed that over a concentration range that is far inferior to commonly used (i.e. 5-10 μM) Sytox Green concentrations[31,36-39], the dye rapidly and dose-dependently leaks into living cells (Fig 1A). A notable effect was consistently detected after only 15 min using just 100 nM of the dye, and massive leaking was observed using 1 μM by 30 min in unstimulated cells (Fig 1A). This was not due to nonspecific staining by Sytox Green of DNA from necrotic cells, since the latter were undetectable at short incubation times, as determined by a lack of PI staining (not shown). Neither did the cell-associated Sytox Green fluorescence result from its staining extracellular DNA, as few NETs were ever observed in unstimulated cells, and accordingly, virtually all the fluorescent signal was still present following DNase I digestion under these conditions (Fig 1B, left panels). In fMLP-

stimulated cells, some NETs were observed using Sytox Green, as expected, but much of the extracellular signal was not associated with extruded DNA, as it was impervious to DNase treatment (Fig 1B, right panels). Thus, the use of Sytox Green entails a large, cell-permeable, nonspecific signal that cannot be easily distinguished from NET-associated fluorescence (unless DNase-treated samples are always processed in parallel).

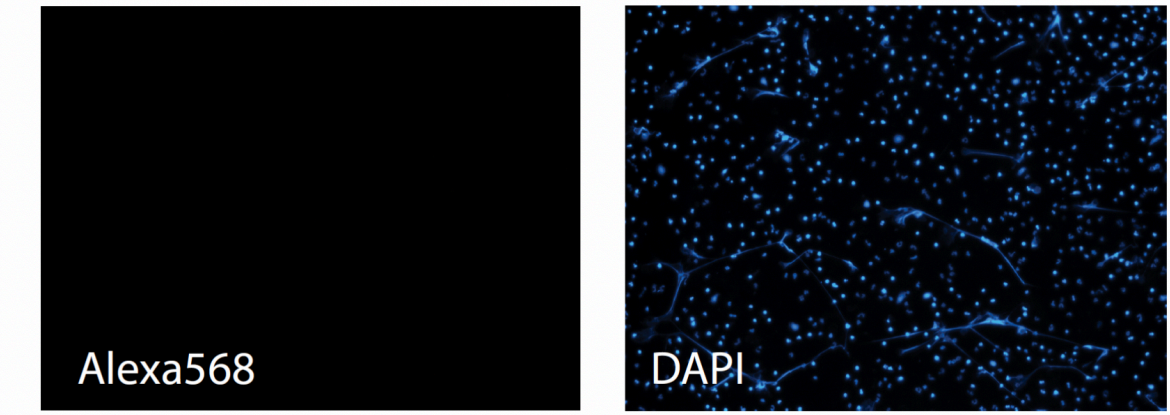


A1 Figure 1. Detection of NETs using Sytox Green in human neutrophils.

(A) Unstimulated neutrophils were cultured in suspension for the indicated times in the presence of increasing concentrations of Sytox Green, prior to the addition of Hoechst 33342 and subsequent fluorescence microscopy analysis of unfixed cells. Total Sytox Green fluorescence values were divided by total Hoechst 33342 fluorescence, to normalize for cell number. Mean ± s.e.m. from duplicate measurements for each experimental condition from a representative experiment, shown on the right at 10X magnification. (B) Neutrophils were cultured for 4 h on poly-L-lysine coated coverslips with the indicated Sytox Green concentrations, in the absence ("ctrl") or presence of 30 nM fMLP. The cells were then digested or not with DNase I (100 U/ml, 30 min, 37°C), then stained

with Hoechst 33342, prior to fluorescence microscopy analysis. A representative experiment is shown (at 40X magnification).

Another common approach is to stain NETs using antibodies directed against associated proteins. However, this can be misleading as several such proteins (*e.g.* MPO, elastase) readily associate with cell membranes upon their release from the cells[40,41]. And indeed, an important fluorescence signal remains near the cell surface following DNase digestion of NETs when the latter are detected using anti-MPO Abs (Fig 2A). This was not due to residual background staining, since no second antibody fluorescence (Alexa 568) was detectable when the experiment was repeated using an isotype control rabbit antibody in substitution for the anti MPO primary antibody (Fig S1).

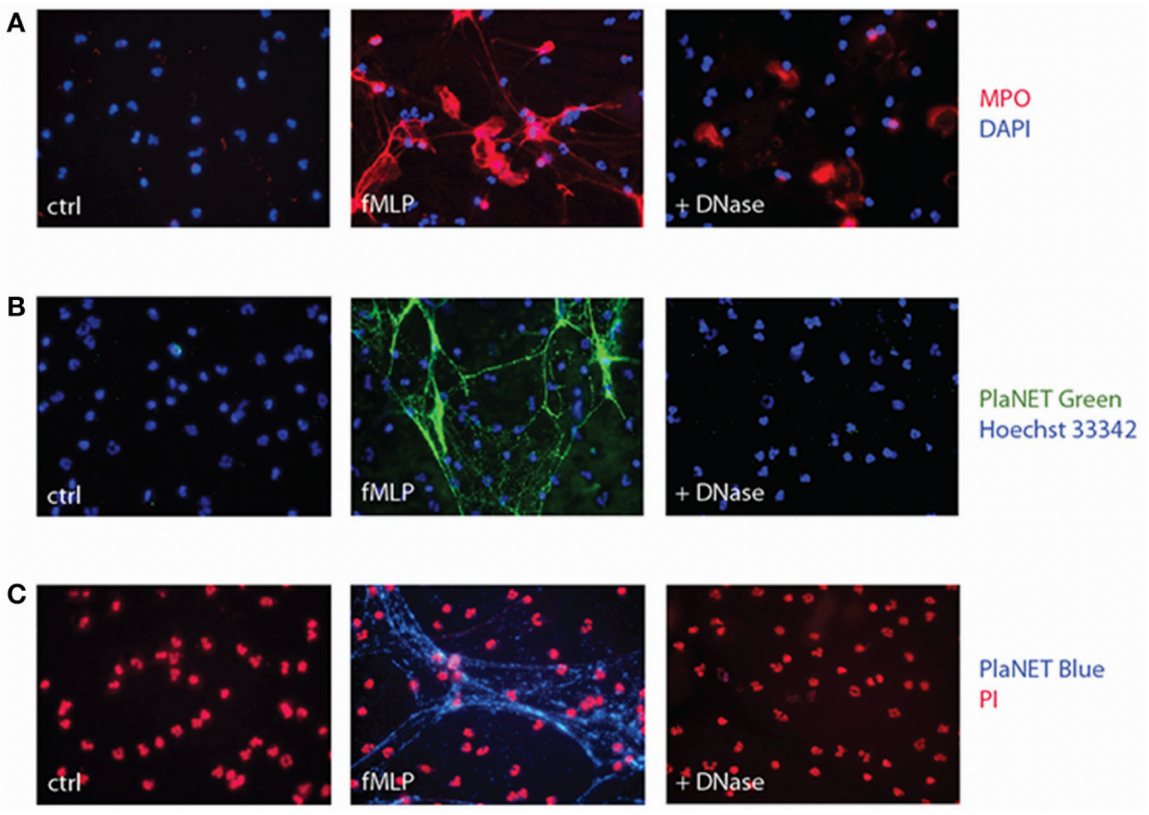


A1 Figure S 1. Isotope control experiment for MPO NET detection.

Neutrophils cultured on poly-L-lysine-coated coverslips were incubated for 4 h in the presence of 30nM fMLP. Fixed cells were blocked with PBS containing 5% normal goat serum and DAPI, incubated with rabbit IgG at the same concentration as the anti-MPO rabbit IgG, and further incubated with goat anti-rabbit-Alexa 568, prior to fluorescent microscopy analysis. A representative experiment is shown (10X magnification).

Thus, commonly used approaches based on the detection of NET-associated granule proteins, or on Sytox Green staining, are fraught with complications when total fluorescence

is counted (as is usually the case), as it includes a substantial (and often predominant) nonspecific signal.

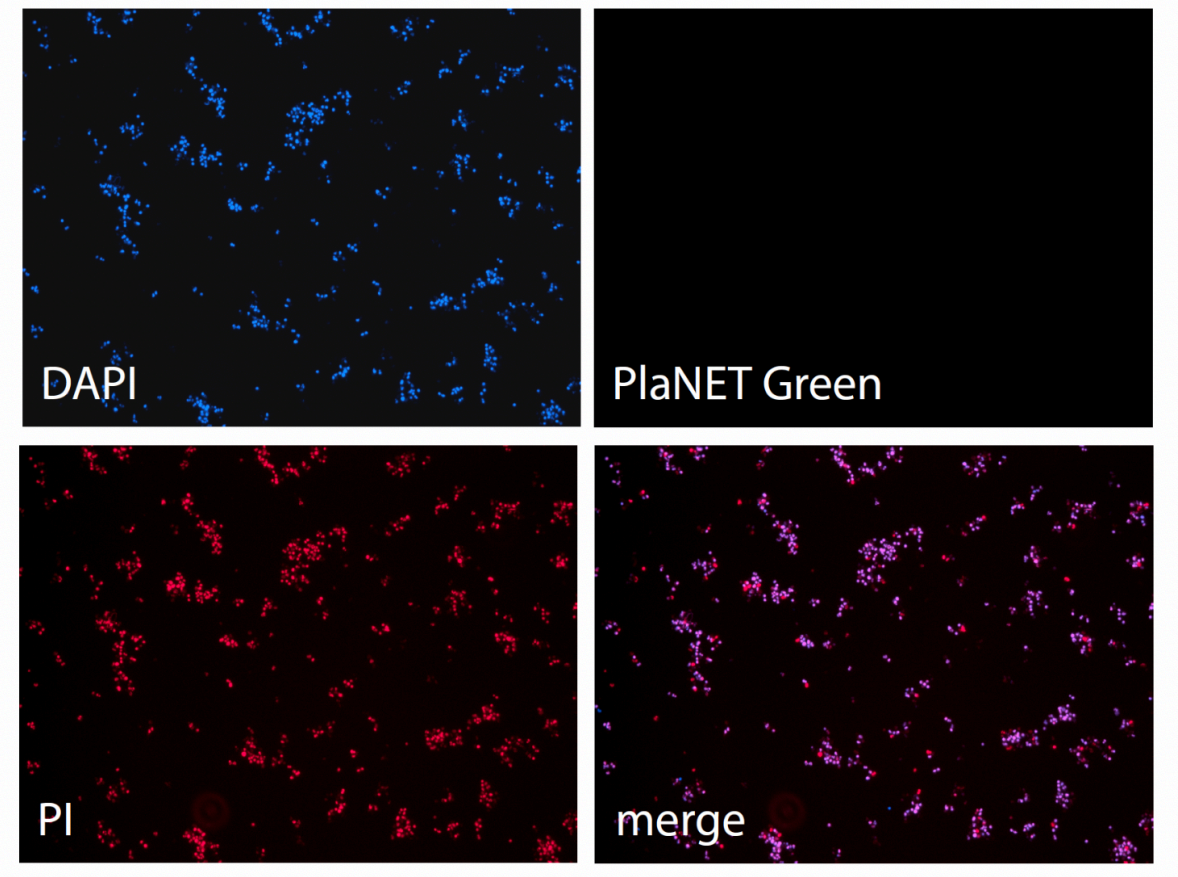


A1 Figure 2. Detection of NETs using MPO or PlaNET reagents in human neutrophils.

(A) Neutrophils cultured on poly-L-lysine-coated coverslips were incubated for 4 h in the absence ("ctrl") or presence of 30 nM fMLP, and digested or not with DNase I, prior to fluorescent microscopy detection of MPO as described in *Methods*. A representative experiment is shown (40X magnification). (B) Neutrophils cultured on poly-L-lysine-coated coverslips were incubated for 4 h in the absence ("ctrl") or presence of 30 nM fMLP, and further incubated in the presence or absence of DNase I (100 U/ml, 30 min, 37°C), prior to fluorescent microscopy detection of NETs using PlaNET Green and Hoechst 33342 counterstaining, as described in *Methods*. A representative experiment is shown (40X magnification). (C) Neutrophils were treated as described in (B); fluorescent microscopy detection of NETs was conducted using PlaNET Blue and propidium iodide counterstaining (5 µM, 20 min), as described in *Methods*. A representative experiment is shown (40X magnification).

In an attempt to overcome this shortcoming, we resorted to PlaNET reagents – newly developed NET detection reagents that are based on fluorescent, chromatin-binding

polymers. As shown in Fig 2B and Fig 2C, PlaNET reagents strongly stain NETs in activated cells, and DNase I digestion completely obliterates the PlaNET reagent signal, thereby showing that is strictly extracellular. In agreement with these findings, PlaNET fluorescnce was also undetectable in cells that were deliberately made necrotic (Fig S2).



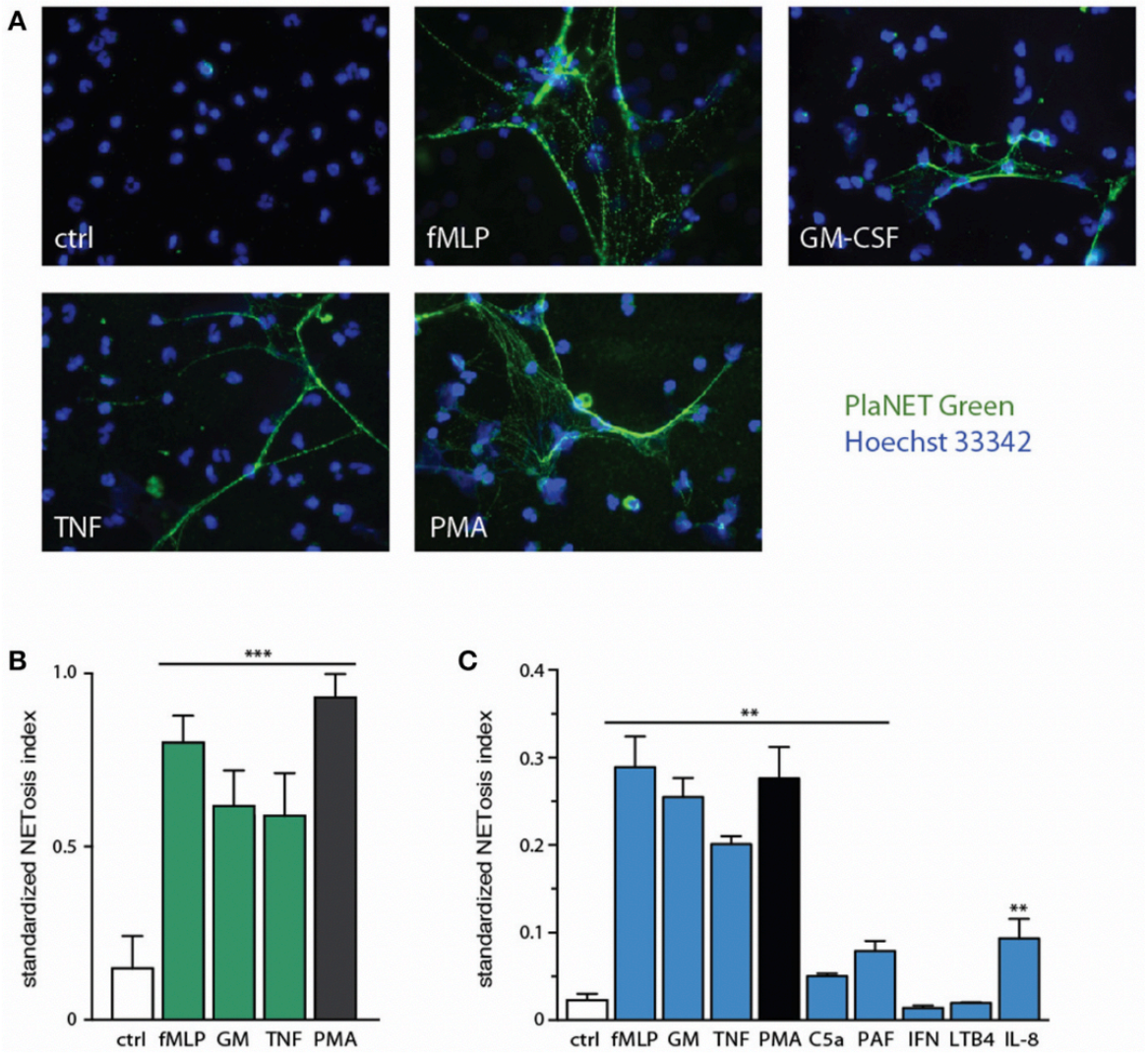
A1 Figure S 2. PlaNET reagents do not stain necrotic cells.

Neutrophils were cultured overnight without stimulation in 6-well plates, and cells that detached from the plates (i.e. necrotic cells) were collected. Necrotic cells were processed following the PlaNET Green procedure described in Methods, with the exception that propidium iodide was included (in addition to DAPI) during the final fixation step, to confirm that the cells were indeed necrotic. A representative experiment is shown (10X magnification).

Thus, measuring total PlaNET reagent fluorescence proves to be a straightforward and specific way of assessing NETosis, independently of necrosis. To ensure optimal

comparisons between samples and experiments, PlaNET fluorescence can be standardized. To this end, we developed a Java-based plug-in (available at http://mcdonaldlab.co.nf/McDonald_Lab/plugin.html) that counts total PlaNET fluorescence and divides it by the number of events (i.e. cells) in the fluorescence channel used for the DNA counterstain, yielding standardized NETosis values.

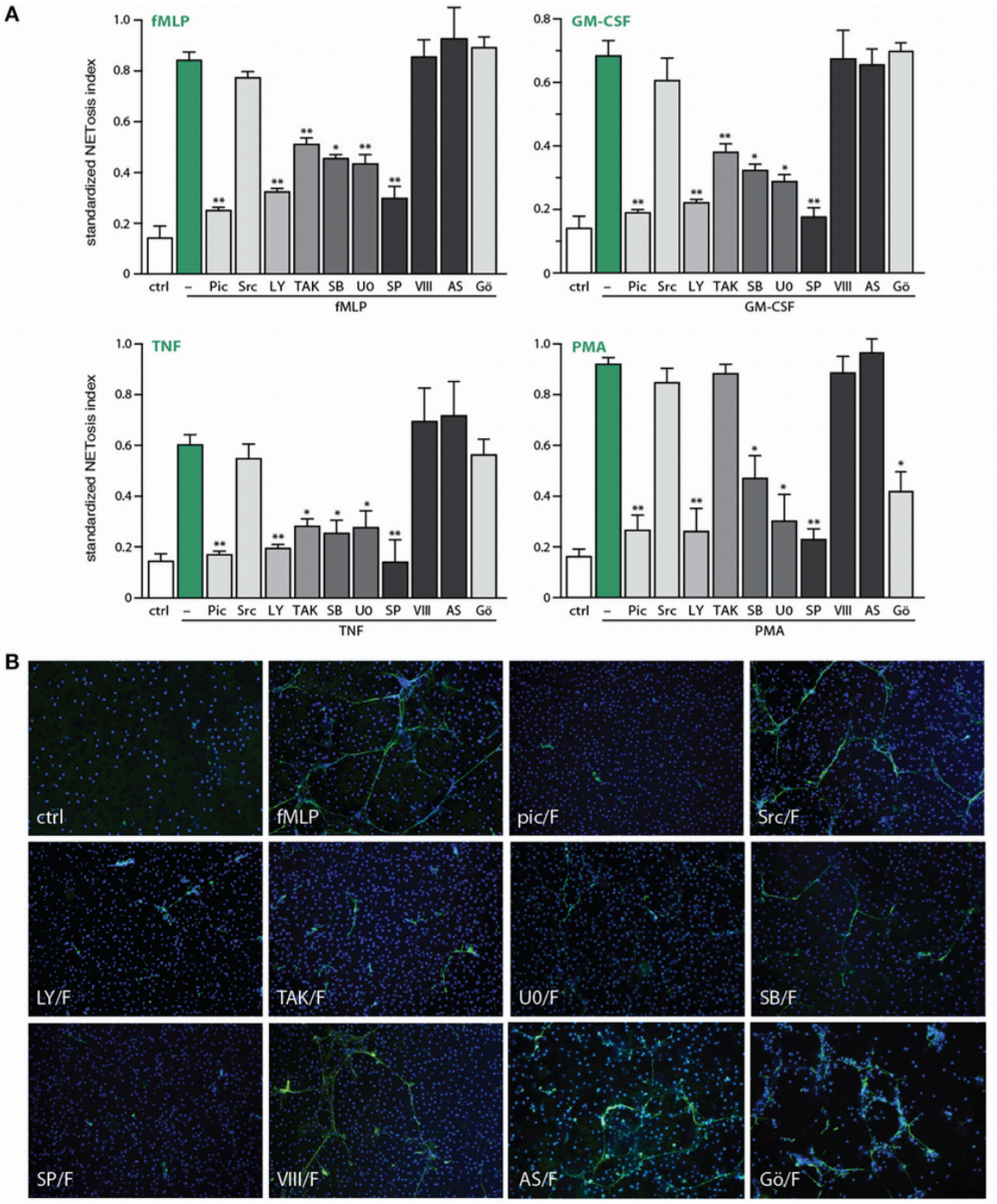
Induction of NET generation by various stimuli, and signaling pathways involved. We used this standardized approach to assess NETosis induction by various neutrophil agonists. As shown in Fig 3A, few unstimulated neutrophils generate NETs after a 4-h incubation period, whereas exposure to various physiological stimuli, or to PMA, results in abundant NET formation. By standardizing NETosis using the Java plug-in, we could compare the relative ability of the stimuli to induce this response; fMLP and PMA stood out as the most potent inducers, with TNFα and GM-CSF following not far behind (Fig 3B), though differences among these stimuli were not found to be statistically significant by one-way ANOVA analysis. Several other physiological stimuli (namely, C5a, PAF, IL-8) were also found to promote NET formation, albeit less potently ($p<0.01$ using one-way ANOVA with Dunnett's correction) than fMLP, GM-CSF, or TNF (Fig 3C); in these experiments, we used PlaNET Blue, as it offers an even better signal-to-noise ratio than PlaNET Green. Finally, other neutrophil stimuli (*e.g.* LTB, IFNγ) failed to stimulate NETosis altogether (Fig 3C).



A1 Figure 3. Relative potency of physiological neutrophil agonists or PMA to induce NETosis.

(A) Neutrophils cultured on poly-L-lysine-coated coverslips were incubated for 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, or 50 nM PMA. NETosis was then assessed using PlaNET Green as described in *Methods*. Representative fields are shown at 40X magnification. (B) Quantitative representation of the above experiments, in which PlaNET Green fluorescence values were standardized according to total cell number (i.e. the number of individual events detected using a cell-permeable nuclear stain), thus yielding a NETosis index. Mean ± s.e.m. from at least 5 independent experiments. ***, $p<0.001$ vs unstimulated cells. (C) Neutrophils were cultured as described above for 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, 50 nM PMA, 30 nM C5a, 50 nM PAF, 100 U/ml IFNγ, 100 nM LTB4, or 10 nM IL-8. Quantitative representation of these experiments, in which PlaNET Blue fluorescence values were standardized as described in (B). Mean ± s.e.m. from 3 independent experiments. **, $p<0.02$ vs unstimulated cells.

We next blocked discrete signaling intermediates using selective inhibitors, prior to stimulation with physiological agonists or PMA, to identify which pathways control NET generation. As shown in Fig 4A, inhibitors of TAK1, MEK, or p38 MAPK potently hindered NETosis in response to GM-CSF, fMLP, or TNFα. In the case of PMA-elicited NETosis, MEK and p38 MAPK inhibition also affected this response, but TAK1 inhibition failed to do so – in keeping with the fact that PMA does not activate TAK1 in neutrophils (our unpublished data). Accordingly, the PMA-induced phosphorylation of ERK, p38 MAPK and Akt were similarly unaffected by TAK1 inhibition (Fig S3). By comparison, inhibition of the Syk, PI3K, and JNK pathways nearly or completely abrogated NET formation in response to all agonists tested (Fig 4A). Finally, inhibition of Src tyrosine kinases consistently failed to interfere with NETosis (Fig 4A). Likewise, inhibition of PKC impaired NETosis elicited by PMA, as expected, but not in response to physiological stimuli (Fig 4A).



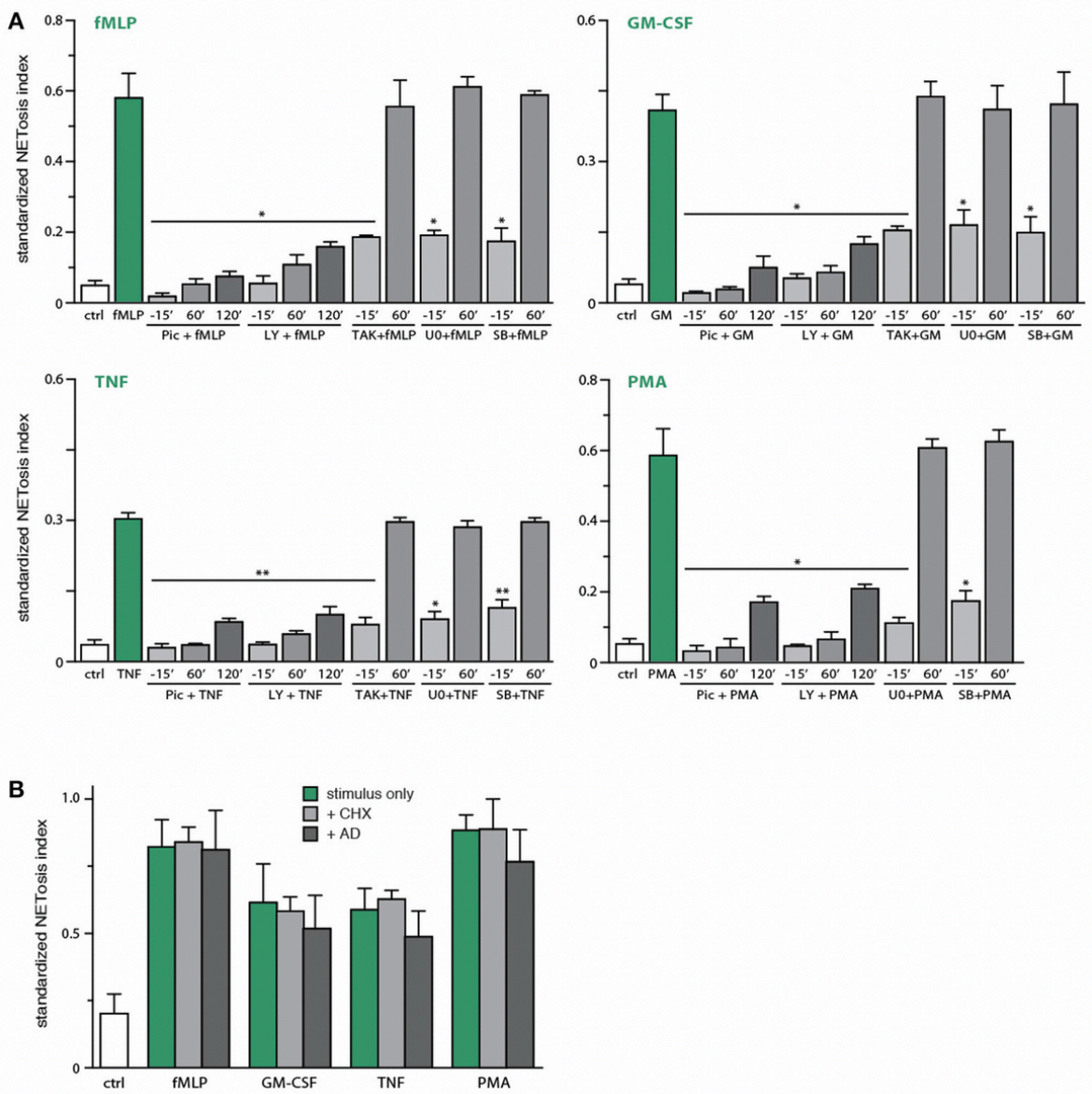
A1 Figure 4. Signaling pathways controlling NETosis induced by physiological neutrophil agonists or PMA.

Neutrophils cultured on poly-L-lysine-coated coverslips were pre-treated (15 min, 37°C) with the following inhibitors or their diluent (DMSO): 10 µM piceatannol (Syk inhibitor); 10 µM SrcI1 (Src family kinase inhibitor); 10 µM LY294002 (PI3K inhibitor); 1 µM (5Z)-7-oxozeaenol (TAK1 inhibitor); 1 µM SB202190 (p38 MAPK inhibitor); 10 µM U0126 (MEK inhibitor); 10 µM SP600125 (JNK inhibitor); 10 µM JNK inhibitor VIII (a different JNK inhibitor); 5 µM AS601285 (a third JNK

inhibitor); 10 μM Gö6976 (a PKC inhibitor). The cells were then further incubated for 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, or 50 nM PMA. NETosis was assessed using PlaNET Green as described in *Methods*. (A) Quantitative representation of these experiments, expressed as NETosis index. Mean ± s.e.m. from at least 3 independent experiments. *, $p<0.05$; **, $p<0.01$ vs stimulus alone. (B) Representative fields for each experimental condition are shown at 10X magnification.

In addition to the above quantitative data, direct microscopic observation revealed qualitative differences between the effects of the various inhibitors towards NETosis. As shown in Fig 4B, inhibition of TAK1, MEK, or p38 MAPK yielded much shorter extracellular chromatin filaments, with little or no interconnectivity, whereas chromatin extrusion *per se* was clearly less affected. This suggests that chromatin extrusion and filament elongation and/or branching could represent distinct steps in the NETosis process. By comparison, cell pretreatment with inhibitors of Syk or PI3K, or the JNK inhibitor, SP600125 (Fig 4B), resulted in little or no chromatin extrusion, suggesting that they prevent this step (or perhaps upstream events). The particular case of JNK inhibition was particularly intriguing, given that some of the stimuli used (*e.g.* fMLP, GM-CSF, PMA) do not promote the phosphorylation of JNK in neutrophils, and can therefore hardly induce neutrophil responses by acting via this kinase. To ensure that the inhibition of NETosis by SP600125 cannot be attributed to nonspecific effects, we used a potent and structurally unrelated JNK inhibitor (called JNK inhibitor VIII). As shown in Fig 4A (last bar) and Fig 4B (last pane), NETosis was unaffected using this second JNK inhibitor, for all stimuli tested. Similarly, a third JNK inhibitor, AS601245, also failed to affect NETosis (Fig 4A). Together, these observations make it very unlikely that JNK participates in controlling NETosis.

Because Syk and PI3K emerged as important upstream intermediates controlling NETosis, we next investigated whether this reflects early or late signaling events, given that NETosis requires 3-4 h to be effectively detected. To this end, kinase inhibitors were either added 15 min before neutrophil stimulation, or 60 to 120 min afterwards. As shown in Fig 5A, NET formation was effectively prevented even when the Syk and PI3K inhibitors were added 120 min post-stimulation, indicating that these pathways are mobilized late in the NETosis phenomenon. Similar results were obtained using SP600125 (Fig S4), though this likely reflects off-target effects, as explained above. By contrast, addition of TAK1, p38 MAPK, or MEK inhibitors 60 to 120 min after neutrophil stimulation failed to affect NET formation (Fig 5A and data not shown), indicating that the contribution of these kinases occurs early in the induction of NETosis. Because stimulated neutrophils express several cytokine and chemokine genes (and release the corresponding proteins) in the same time frame required for NET formation, and since several such products are NET inducers, we also examined whether gene transcription or *de novo* protein synthesis might participate in NETosis. As shown in Fig 5B, the blockade of transcription (using actinomycin D) or of protein synthesis (using cycloheximide) failed to alter NETosis elicited by physiological stimuli or PMA.

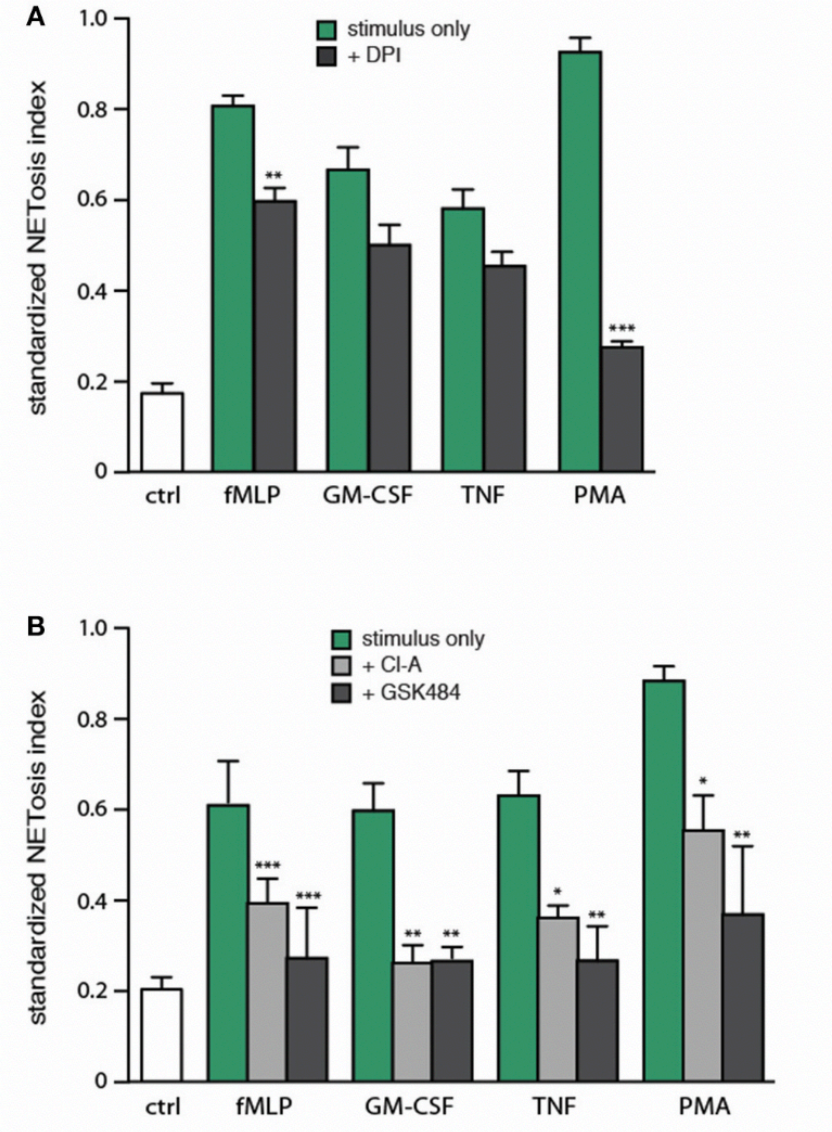


A1 Figure 5. Identification of early and late processes underlying NET generation.

(A) Neutrophils cultured on poly-L-lysine-coated coverslips were treated either before or after stimulation for the indicated times with the following inhibitors or their diluent (DMSO): 10 µM piceatannol ("pic", Syk inhibitor); 10 µM LY294002 (PI3K inhibitor); 1 µM (5Z)-7-oxozeaenol (TAK1 inhibitor); 1 µM SB202190 (p38 MAPK inhibitor); 10 µM U0126 (MEK inhibitor). The cells were also stimulated for 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, or 50 nM PMA. NETosis was then assessed using PlaNET Green as described in *Methods*. Quantitative representation of these experiments, expressed as NETosis index. Mean ± s.e.m. from 3 independent experiments. *, $p<0.05$; **, $p<0.01$ vs stimulus alone. (B) Neutrophils cultured on poly-L-lysine-coated coverslips were pre-treated (15 min, 37°C) with 20 µg/ml cycloheximide, 5 µg/ml actinomycin D, or their diluent (DMSO), prior to a further incubation of 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, or 50 nM PMA. NETosis was then assessed using PlaNET Green as described in *Methods*. Quantitative representation

of these experiments, expressed as NETosis index. Mean ± s.e.m. from at least 3 independent experiments.

Involvement of endogenous ROS and PAD4 in NET generation. Because NETosis can be induced by stimuli that are ineffective ROS inducers[11,13], it would seem that under some circumstances, NETosis must take place independently of ROS production. To investigate the issue, neutrophils were pretreated with DPI (a NADPH oxidase inhibitor), prior to stimulation. As expected, PMA-elicited NETosis was almost entirely dependent on NADPH oxidase activation (Fig 6A). NET formation in response to fMLP was also significantly affected by DPI, but to a far lesser extent (Fig 6A). In contrast, TNF- or GM-CSF-induced NETosis were not significantly inhibited by DPI (Fig 6A). These results show that the phenomenon is largely ROS-independent in response to various physiological agonists.



A1 Figure 6. Involvement of endogenous ROS and PAD4 in NET generation.

(A) Neutrophils cultured on poly-L-lysine-coated coverslips were pre-treated (15 min, 37°C) with 10 µM DPI or its diluent (DMSO), prior to a further incubation of 4h in the absence ("ctrl") or presence of 30 nM fMLP, 1 nM GM-CSF, 100 U/ml TNFα, or 50 nM PMA. NETosis was then assessed using PlaNET Green as described in *Methods*. Quantitative representation of these experiments, expressed as NETosis index. Mean ± s.e.m. from at least 5 independent experiments. **, $p<0.01$; ***, $p<0.0001$ vs stimulus alone. (B) Neutrophils cultured on poly-L-lysine-coated coverslips were pre-treated (15 min, 37°C) with 10 µM chloraminidine ("Cl-A", a general PAD inhibitor), 10 µM GSK484 (a PAD4 inhibitor), or their diluent (DMSO), prior to a further incubation of 4h in the absence ("ctrl") or presence of the above stimuli, followed by determination of the NETosis index. Mean ± s.e.m. from at least 4 independent experiments. *, $p<0.04$; **, $p<0.01$; ***, $p<0.001$ vs stimulus alone.

Arginine deimination has also been proposed to participate in NETosis, insofar as PAD inhibition or deficiency hinders NETosis[16-19]. To further investigate the role of PAD4 in NETosis, we pretreated neutrophils with either chloraminidine (a general PAD inhibitor) or GSK484 (a selective PAD4 inhibitor)[22], prior to stimulation with physiological agonists or PMA. Both inhibitors abrogated the citrullination of histone H3, as expected (Fig S5). As shown in Fig 6B, both chloraminidine and GSK484 strongly inhibited NET formation under all conditions tested. NETosis therefore appears to involve PAD4 in human neutrophils. Worthy of note is that chloraminidine did not retain its potency for more than a month after having been prepared; care should therefore be taken to use only freshly-prepared stocks of this inhibitor.

Discussion

Since its discovery a dozen years ago, NETosis has emerged as a major neutrophil functional response, and its study represents an area of intense ongoing research. Whereas diverse methods have been used to assess NETosis, most suffer from significant drawbacks, in particular the inclusion of a false positive signal that can often be predominant. In this study, we used newly developed fluorescent reagents that allow a streamlined, reliable, and standardized assessement of NET formation. This allowed us to quantitatively compare NETosis induction by various physiological stimuli, to shed a new light on the signaling pathways involved, and to unveil some of the underlying mechanisms.

Using PlaNET reagents, we showed that NETosis can be measured specifically, insofar as the entire signal disappears following DNase I digestion – unlike widely used assays based on the detection of NET-associated proteins (such as myeloperoxidase or elastase), in which a strong fluorescent signal remains after DNase treatment, possibly reflecting the

propensity of several neutrophil granule proteins to strongly associate with cell membranes upon their release from the cells[40,41]. Likewise, PlaNET reagents do not enter live or necrotic cells, unlike widely used detection reagents such as Sytox Green, which do so rapidly and in a concentration-dependent manner. A workaround for the shortcomings of conventional approaches to assess NETosis would be to always carry out experiments in the presence and absence of DNase digestion, even though this would automatically double the size and cost of any experiment. Thus, the specificity of PlaNET reagents represents a major advantage over commonly used approaches to study NETosis. This being said, PlaNET reagents are not without some drawbacks, as we found that they are not completely suitable for kinetic assessment of NETosis in microtiter plate assays. This is because neutrophils co-incubated with PlaNET reagents somehow ingest some of the polymers, resulting in a non specific signal. This could be prevented by including PMSF in the culture medium, but whether incubation of living cells in the continued presence of this inhibitor might affect other processes would be a potential concern. For this reason, we would not advise using PlaNET reagents for kinetics studies in a plate reader.

We also developed a simple Java plug-in to standardize NETosis measurement based on the total number of neutrophils, and found that doing so helps minimize both intra-experiment and inter-donor variation. In this regard, another group recently reported similar benefits from standardizing NET detection[42], though they used Sytox Green which we (and other investigators) found to enter the cells to a significant degree. Another important benefit of standardizing is that it allowed us to quantitate the relative ability of various stimuli to elicit NETosis, and the extent to which various inhibitors affect the phenomenon. Thus, we found that fMLP, PMA, TNF, and GM-CSF are potent inducers; IL-8 are also a good inducer, but comparatively weaker; finally, C5a and PAF proved to be weak stimuli.

Conversely, certain neutrophil activators were found to induce little or no NET formation (*e.g.* LTB4, IFNγ), showing that not all neutrophil stimuli act as NET inducers.

Pharmacological blockade of various signaling pathways revealed that several kinases (*e.g.* Syk, PI3K, TAK1, p38 MAPK, MEK) profoundly affect NETosis. Although Syk and PI3K inhibitors were consistently more effective than the ones for TAK1 and the MAP kinases, this difference was not found to be statistically significant by one-way ANOVA analysis. By contrast, inhibition of Src tyrosine kinases consistently failed to interfere with NETosis. For physiological stimuli such as TNFα, fMLP, or GM-CSF, our data are consistent with our previous findings, which showed that they can all signal through the TAK1-MEK or TAK1-p38 axes in neutrophils[43,44]. Conversely, PMA does not activate TAK1 in these cells (our unpublished data), and accordingly, TAK1 inhibition had no significant effect on PMA-elicited NETosis. In the particular case of JNK inhibition, we found it surprising that in TNF-stimulated neutrophils, TAK1 inhibition of NETosis was less pronounced than that exerted by the widely used JNK inhibitor, SP600125, given that TNF activates JNK downstream of TAK1 in these cells[43]. Likewise, we found it peculiar that SP600125 should abrogate NETosis even when ineffective JNK activators (*e.g.* PMA, fMLP, GM-CSF) were used as stimuli. In this regard, SP600125 is known to exert nonspecific effects towards 13 other kinases[45], and to even inhibit PI3K as effectively as wortmannin in mast cells[46]. The latter observation is particularly alarming, in view of how potently PI3K inhibition prevents NETosis, as shown herein and in other studies[47,48]. Together, the above considerations cast a serious doubt as to whether SP600125 affects NETosis through JNK inhibition, as opposed to off-target actions. To settle the matter, we resorted to very selective, structurally unrelated JNK inhibitors (i.e. JNK inhibitor VIII and AS601285), which both failed to affect NETosis in response to all

stimuli tested. This is compelling evidence that JNK does not control the phenomenon. This conclusion contrasts with a recent study, in which SP600125 and TCSJNK6o (also known as JNK inhibitor VIII) obliterated LPS-induced NETosis while they only minimally affected the phenomenon in PMA-treated cells, using a Sytox Green-based NET assay[39]. This discrepancy between their data and ours is not easy to resolve, especially since we used similar concentrations of PMA and JNK inhibitors. However, the experimental procedures differ significantly. Because we thoroughly controlled for false positives (i.e. DNAse-insensitive or necrotic cell-derived signals) when assessing NETosis, and because we used three different JNK inhibitors, we stand by our conclusion, that JNK does not control NETosis in response to several classes of neutrophil stimuli.

Previous reports had already shown that Syk and PI3K are crucial for PMA-induced NETosis[49,47,48]. Our data confirm these observations, but more importantly, reinforce their significance by demonstrating that this is also true of NETosis triggered by physiological stimuli. We further showed that Syk and PI3K do so by acting upon chromatin extrusion or upstream processes, and that this involves late signaling events in NETosis (occurring at about 120 min of stimulation). This is a major new observation. The nature of the late processes affecting NETosis, however, remains elusive. We could exclude newly-made cytokines and chemokines as potential candidates, even though they are produced in the right time frame and are potent NET inducers, since neither cycloheximide nor actinomycin D were found to affect NETosis in response to any of the stimuli used. Khan and colleagues similarly observed that cycloheximide does not affect NET formation in response to PMA or ionomycin, but reported that actinomycin D blocks the phenomenon[50]. However, another group[51] found no effect of either cycloheximide or actinomycin D on NETosis elicited by PMA or *C. albicans*, in full agreement with our data.

Thus, while it is quite clear that *de novo* protein synthesis does not contribute to NET formation, there is growing evidence for a similar conclusion in the case of gene transcription. In contrast to Syk and PI3K, other kinases (i.e. TAK1, p38 MAPK, MEK) seem to control early events (within the first 15 min) of the NETosis process, and to influence the length and degree of branching of extruded chromatin filaments, as opposed to chromatin extrusion itself. Studies are in progress to further define each aspect of the NETosis phenomenon.

We finally revisited the issue of whether NETosis is a ROS-dependent process. In this regard, it is noteworthy that the bulk of available data has been obtained using powerful NADPH oxidase activators, such as PMA or bacterial phagocytosis. However, NETosis has also been observed in response to stimuli that are ineffective ROS inducers, such as ionomycin, GM-CSF, TNFα, or IL-1[11,13]. We confirmed herein that PMA-induced NETosis is indeed ROS-dependent (and PKC-dependent), but also show that NETosis occurring in response to various classes of physiologic stimuli is largely unaffected by inhibition of the NADPH oxidase or of PKC. These observations agree well with recent studies, which have shown that NETosis can take place in a ROS-independent fashion following neutrophil exposure to uric acid, mercury, nicotine, immune complexes, or endotoxin[52,30,20,42,53]. Our data therefore adds to the mounting evidence that endogenous ROS are far from essential for NETosis, though they can certainly contribute to the process under some circumstances. By contrast, we found that NETosis occurring in response to all stimuli investigated (including PMA) depends on PAD4. Previous reports had reached a similar conclusion, based on the fact that chloraminidine prevents NET formation in response to calcium ionophores, bacteria, IL-8, PMA, or even nicotine[16,20,21]. However, chloraminidine is a general PAD inhibitor that does not discriminate between PAD

isoforms, and both PAD2 and PAD4 have been observed on NETs in a pathological setting in humans[15]. Mouse studies have suggested that PAD4 might be the relevant molecule, insofar as NETosis does not occur in PAD4-deficient animals[17-19], whereas PAD2 is dispensable[54]. Our finding, that a selective PAD4 inhibitor prevents NETosis as well as chloraminidine in human neutrophils, represents the first demonstration that PAD4 is also the relevant PAD isoform in humans. Thus, it appears that NETosis is a PAD4-dependent phenomenon that may also require endogenous ROS, depending on the stimulatory conditions. This represents a significant shift in how NETosis has heretofore been viewed.

In summary, we describe a reliable and specific appoach to assess NETosis. This allowed us to determine the relative potency of various physiologic NET inducers; to extend our knowledge of the signaling pathways involved, and of how they affect early or late stages of the phenomenon; and to identify PAD4 as required for NETosis, whereas ROS do not necessarily contribute to this response. In view of the involvement of NETs in several pathologies, our findings reveal potential molecular targets that could be exploited for therapeutic intervention. In this regard, inhibitors of several such molecules are already in phase I/II clinical trials[55-60].

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ARTICLE 2

Cytokine production and NET formation by monosodium urate-activated human neutrophils involves early and late events, and requires upstream TAK1 and Syk

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Running title: TAK1 and Syk control MSU responses in neutrophils

Keywords: neutrophils, signaling, cytokines, NETs, transcription factors

Authorship

Contribution: OT carried out the experiments for most aspects of the paper, compiled most the data, and wrote the first draft; TZM compiled and analyzed the gene microarray data; MI and SSP carried out the initial experiments for this project; CMD provided conceptual input; PPMcD designed the research, mentored the other authors, and wrote the final version of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Abstract

Objectif. La goutte est une maladie fréquente et invalidante déclenchée par le dépôt de cristaux d'urate monosodique (MSU) dans les articulations, lesquelles sont massivement infiltrées par des neutrophiles. Bien que certains éléments de signalisation utilisés par le MSU chez les neutrophiles aient été décrits, le portrait global demeure très incomplet. De façon semblable, l'impact des divers événements de signalisation sur les réponses cellulaires est loin d'être complètement élucidé. Dans cette étude, nous avons examiné les modifications génomiques et protéomiques déclenchées par le MSU chez les neutrophiles humains, ainsi que le rôle de différentes voies de signalisation dans plusieurs réponses fonctionnelles importantes de ces cellules.

Méthodes. Les neutrophiles humains ont été stimulés avec du MSU et une analyse génétique par micropuces a été réalisée; et les protéines correspondant aux gènes les plus induits ont aussi été investiguées. Les voies de signalisation affectées par le MSU et leur relation avec les kinases en amont ont été examinées par immunobuvardage. Les facteurs de transcription activés par le MSU ont eux aussi été étudiés. L'impact des intermédiaires de signalisation sur la production de cytokines et la formation de NETs (*Neutrophil Extracellular Traps*) a été quantifié, respectivement, par ELISA et microscopie à fluorescence.

Résultats. Nous rapportons pour la première fois que les neutrophiles peuvent sécréter du CCL4 en réponse au MSU. De plus, nous avons trouvé que les facteurs de transcription NF-κB, CREB et C/EBP sont activés tardivement par les cristaux de MSU, et qu'au moins le premier est impliqué dans la production de chimiokines. Enfin, nous démontrons que les MAPKs et Akt qui sont activés par le MSU chez les neutrophiles, sont sous le contrôle de TAK1 et de Syk, et qu'ils participent à la production de cytokines et à la formation de NETs.

Conclusion. Les résultats actuels dévoilent des cibles thérapeutiques potentiellement importantes pour la goutte.

Abstract

Objective. Gout is a prevalent and incapacitating disease triggered by the deposition of monosodium urate (MSU) crystals in joints, which are also massively infiltrated by neutrophils. Though some of the signaling events mobilized by MSU in neutrophils have been described, the picture remains fragmentary. Likewise, the impact of these signaling events on cellular responses is incompletely understood. In this study, we examined genomic and proteomic changes triggered by MSU in neutrophils, as well as the role of various signaling pathways in prominent functional responses.

Methods. Human neutrophils were cultured with MSU and gene microarray analysis was performed; and proteins corresponding to the genes most induced were also monitored. Signaling pathways mobilized by MSU and their relationship with upstram kinases were investigated by immunoblot. Ttranscription factors activated by MSU were also likewise studied. The impact of signaling intermediates on cytokine production and neutrophil extracellular trap (NET) formation was quantified by ELISA and fluorescence microscopy, respectively.

Results. We report for the first time that neutrophils can secrete CCL4 in response to MSU. Accordingly, we found that transcription factors NF-κB, CREB, and C/EBP are belatedly activated by MSU crystals, and at least the former is involved in chemokine generation. Moreover, we show that MAPKs and Akt are activated by MSU in neutrophils, that they are under the control of TAK1 and Syk, and that they participate in cytokine generation and NETosis.

Conclusion. The current findings unveil potentially important therapeutic targets for gouty arthritis.

Introduction

Gout is a prevalent disease (about 1 in 50 people will develop it over a lifetime) that is very painful and incapacitating (recurring gout attacks can cause permanent joint damage). One clear distinction between gout and other arthritides is that its causative agent is known. Deposition of insoluble monosodium urate (MSU) crystals in the joint triggers an acute inflammatory reaction that is partially initiated and driven by neutrophils. Accordingly, the main mediators detected in the synovial fluid of gouty joints (i.e. IL-1b, IL-6, CXCL8, CCL3, TNFα), whether in humans(1) or in animal models(2), can all be secreted by neutrophils. More compellingly, neutrophil depletion suppresses the inflammatory response to MSU in canine joints (3, 4). Likewise, colchicine, an effective (but poorly tolerated) treatment for acute gout, potently inhibits numerous neutrophil functions (5). Together, these observations leave little doubt that neutrophils and their products represent important elements in the pathogenesis of gout.

Interactions between neutrophils and MSU crystals are known to elicit several responses. One of the first to be documented was the production of reactive oxygen species (ROS) and the concurrent release of anti-microbial peptides and proteolytic enzymes (6, 7). Neutrophils were also shown to synthesize and release the potent neutrophil chemoattractant, leukotriene B4, as well as other neutrophil chemotactic factors in response to MSU (8-11). Likewise, MSU-activated neutrophils can secrete cytokines and chemokines in response to MSU,

namely IL-1β, IL-1ra, and CXCL8 (12-14). Neutrophils stimulated with MSU crystals also display a significantly delayed apoptosis (15, 16), which presumably contributes to their increased recruitment and persistence during active gouty inflammation. Finally, the ability of MSU to elicit the generation of neutrophil extracellular traps (NETs) was recently reported (17, 18).

Because of the numerous actions of MSU crystals towards neutrophils, several studies have focused on the underlying mechanisms; despite this however, our knowledge of the signaling pathways being mobilized remains fragmentary. It has been shown, for instance, that MSU rapidly triggers the phosphorylation of several neutrophil proteins on tyrosine residues, and that accordingly, tyrosine kinases such as Syk and members of the Src family are rapidly activated by the crystals in these cells (19, 20). Other kinases, namely conventional PKCs, were reported to be activated by MSU in neutrophils, and there is evidence that these PKCs can associate with Syk, resulting in its phosphorylation and interaction with PI3Ks (21, 22). Finally, studies involving pharmacological inhibitors have indicated that Src family kinases, Syk, and PI3Ks act as key signaling molecules for MSU-elicited degranulation, ROS production, generation of chemotactic activity, and NETosis in neutrophils (9, 17, 19, 22).

In view of the prevalence of gouty arthritis and of the neutrophil involvement in its pathogenesis, a better understanding of both MSU-elicited responses and of their molecular bases is clearly needed. In this regard, our previous work has provided several potential clues, insofar as we have shown the crucial involvement of TAK1, MAPKs, PI3K, and Syk in

cytokine generation, delayed apoptosis, and NETosis in response to several physiological neutrophil stimuli (23-27). Under the same stimulatory conditions, we have also established that several transcription factors (*e.g.* NF-κB, C/EBP, CREB) drive cytokine production in neutrophils (23, 26, 28, 29). These observations raise the possibility, that some of the same kinases (in addition to Syk and PI3K) and transcription factors similarly control MSU-elicited responses. In this study, we examined the genomic and proteomic changes triggered by MSU in neutrophils, as well as the role of various signaling pathways in these and other functional responses. We now report for the first time that neutrophils can secrete CCL4 in response to MSU. Accordingly, we found that transcription factors NF-κB, CREB, and C/EBP are belatedly activated by MSU crystals, and at least the former is involved in cytokine generation. Moreover, we show that MAPKs are activated by MSU in neutrophils, that they are under the control of TAK1 and/or Syk, and that they participate in cytokine generation and NETosis.

Materials and Methods

Antibodies and reagents. Antibodies against P-Akt (#4060), P-ERK (#9101), P-p38 (#9212), P-Src (#2101), P-Syk (#2711), P-C/EBPβ (#3084), P-CREB (#9191), P-RelA (#3031), IκBζ (#9244) and MAP3K8 (#4491) were all from NEB-Cell Signaling (Danvers, MA, USA); antibodies against IκB-α (sc-371) and β-actin (sc-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ficoll-Paque Plus was from GE Biosciences (Baie d'Urfé, Qc, Canada); endotoxin-free (< 2 pg/ml) RPMI 1640 was from Wisent (St-Bruno, Qc, Canada). Monosodium urate crystals were from Cayman Chemical (Ann Arbor, MI, USA); recombinant human cytokines were from R&D Systems (Minneapolis, MN, USA); UltraPure LPS (from *E. coli* 0111:B4) was from InvivoGen (San Diego, CA, USA).

Actinomycin D, cycloheximide, culture-grade dimethyl sulfoxide (DMSO), N-formyl-leucyl-methionyl-phenylalanine (fMLP), and phenylmethanesulphonyl fluoride (PMSF) were from Sigma-Aldrich (St. Louis, MO, USA). Diisopropyl fluorophosphate (DFP) was from Bioshop Inc. (Burlington, Ont., Canada). The protease inhibitors, aprotinin, 4-(2-aminomethyl)benzenesulfonyl fluoride (AEBSF), leupeptin, and pepstatin A, were all from Roche (Laval, Qc, Canada). Kinase inhibitors and fluorescent probes were purchased through Cedarlane Labs (Missisauga, Canada). PlaNET Blue reagent was from Sunshine Antibodies (https://sunshineantibodies.com/planet-002.html). All other reagents were of the highest available grade, and all buffers and solutions were prepared using pyrogen-free clinical grade water.

Cell isolation and culture. Neutrophils were isolated from the peripheral blood of healthy donors, following a protocol that was approved by an institutional ethics committee (Comité d'éthique de la recherche du CIUSS de l'Estrie-CHUS). The entire procedure was carried out at room temperature and under endotoxin-free conditions, as described previously (30). Purified neutrophils were resuspended in RPMI 1640 supplemented with 5% autologous serum, at a final concentration of 5 x 10^6 cells/ml (unless otherwise stated). As determined by Wright staining and FACS analysis, the final neutrophil suspensions contained fewer than 0.1% monocytes or lymphocytes; neutrophil viability exceeded 98% after up to 4 h in culture, as determined by trypan blue exclusion and by Annexin V/propidium iodide FACS analysis.

Immunoblots. Samples were prepared, electrophoresed, transferred onto nitrocellulose, and processed for immunoblot analysis as previously described (26, 31).

Immunoprecipitations and in vitro kinase assays. The procedure used was exactly as described(26).

RNA extractions, real-time PCR analyses, and gene microarray analyses. Procedures and primers used are exactly as described (28). When samples were prepared for gene microarray analysis, total RNA from 5 x 10^7 neutrophils was isolated as described (28), purified using a Qiagen RNeasy MinElute cleanup kit, and processed for gene microarray analysis using the Affymetrix Human Gene 2.0 ST chip (Génome Québec, Montréal, Qc, Canada).

Gene microarray analyses. Total RNA from 5 x 10^7 neutrophils was isolated as described (28), purified using a Qiagen RNeasy MinElute cleanup kit, and processed for gene microarray analysis using the Affymetrix Human Gene 2.0 ST chip (Génome Québec, Montréal, Qc, Canada). *ELISA analyses.* Neutrophils (3 x 10^6 cells/600 µl) were cultured in 24-well plates at 37°C under a 5% CO_2 atmosphere, in the presence or absence of stimuli and/or inhibitors, for the indicated times. Culture supernatants, as well as the corresponding cell pellets, were carefully collected, snap-frozen in liquid nitrogen, and stored at -80°C. Samples were analyzed in ELISA using commercially available capture and detection antibody pairs (R&D Systems, BD Biosciences).

NETosis assays. The procedure used was exactly as described (27).

Data analysis. All data are represented as the mean ± SEM. Statistical differences were analyzed by Student's t test for paired data using Prism 7 software (GraphPad Software, San Diego, CA, USA).

Results

Transcriptomic changes elicited by MSU in neutrophils, and its consequences on cognate proteins. We first revisited the issue of the genes induced by MSU crystals in neutrophils, a response that has not been systematically investigated to date. The cells were initially cultured for 1 h with MSU, in an effort to detect immediate-early genes, and total RNA was processed for gene microarray analysis. Disappointingly, no transcript was induced by more than 1.8 fold; likewise, no transcript was reduced by more than 2 fold (data not shown). Thus, transcriptomic changes exerted by MSU at early stimulation times are modest at best. We repeated these experiments using neutrophils stimulated with MSU for 3 h, to determine whether gene expression changes are more pronounced at later times. As shown in Fig S1, most genes examined exhibited changes in expression that were less than 3 fold.

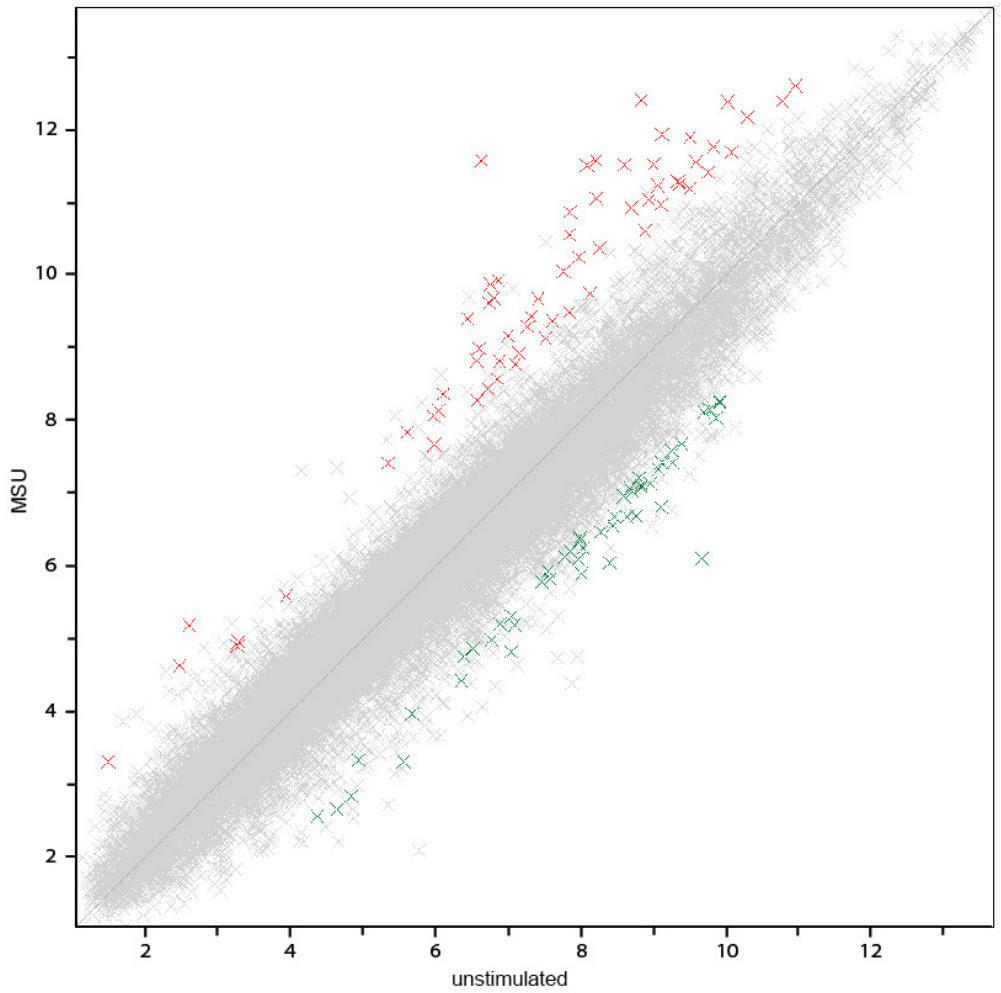

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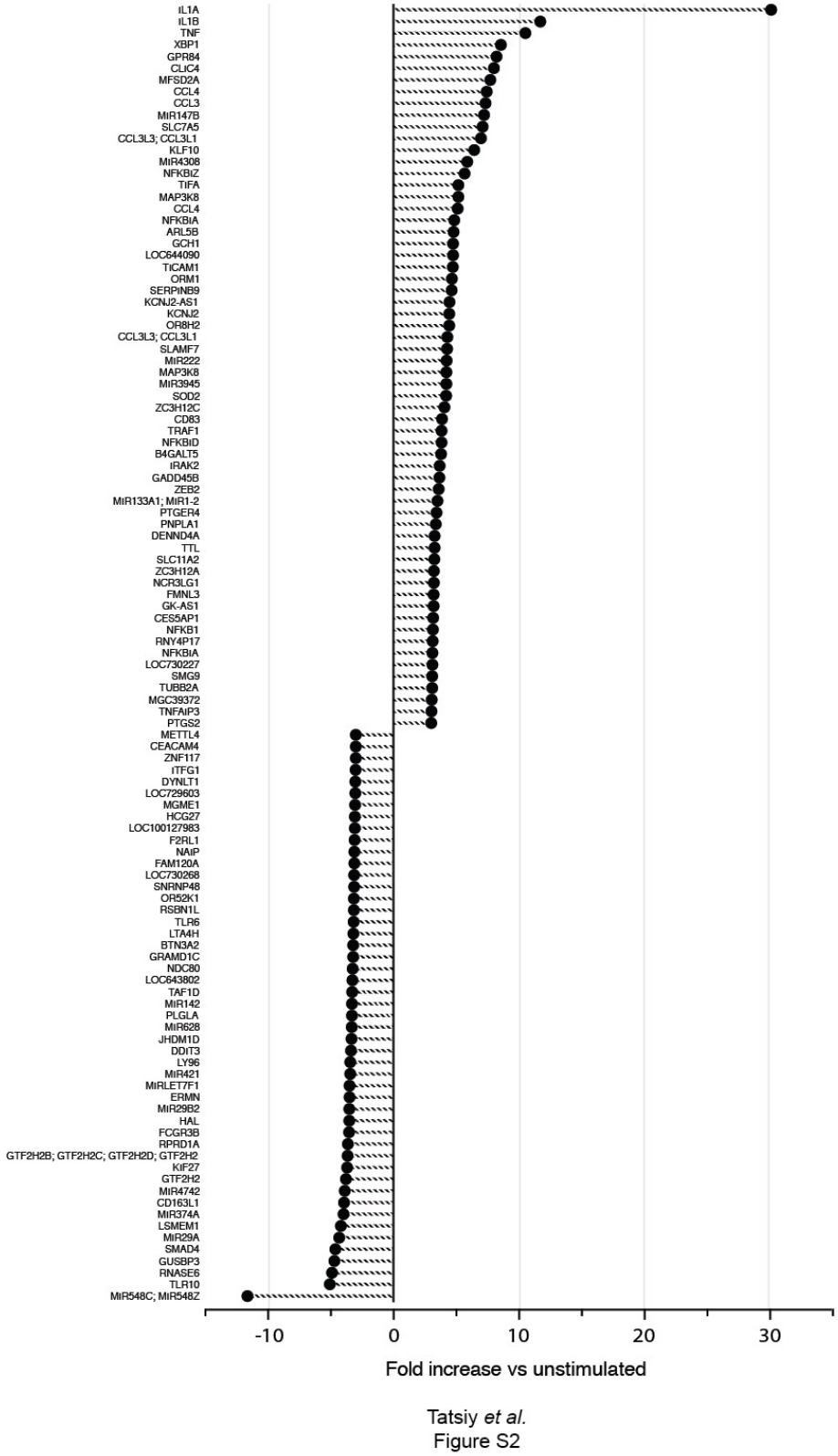
Figure S1

A2 Figure S 1. Genomic changes elicited by MSU in human neutrophils.

Cells were stimulated with 1 mg/ml MSU (or diluent control) for 3 h at 37°C; total RNA was then extracted and 3 µg per experimental condition was pooled among 3 independent experiments and processed for gene microarray analysis. The scattergraph depicts the expression of various transcripts, relative to unstimulated controls. Colored crosses represent transcripts that are modulated 3-fold or more, for which the corresponding protein is known.



A2 Figure S 2. Genomic changes elicited by MSU in human neutrophils.

Cells were stimulated and samples processed as decribed in Fig S1, and for genes with known corresponding proteins whose expression was modulated 3-fold or more, the extent of modulation is depicted.

Despite this, several genes encoding inflammatory products were detected, whose expression was induced 3-fold or more (versus unstimulated cells). These included IL-1α/β and CXCL8, as already reported (12-14), but also included transcripts that had not yet been observed to be induced in response to MSU, such as TNFα, CCL4, and Tpl2/MAP3K8 (Fig S2). Other genes were similarly induced, whose products are however unknown (Fig S2). When we validated these results by qPCR, we confirmed that the TNFα, IL-1β, CXCL8, CCL4, MAP3K8, and IκBζ genes were indeed strongly induced by MSU in human neutrophils (Fig 1A).

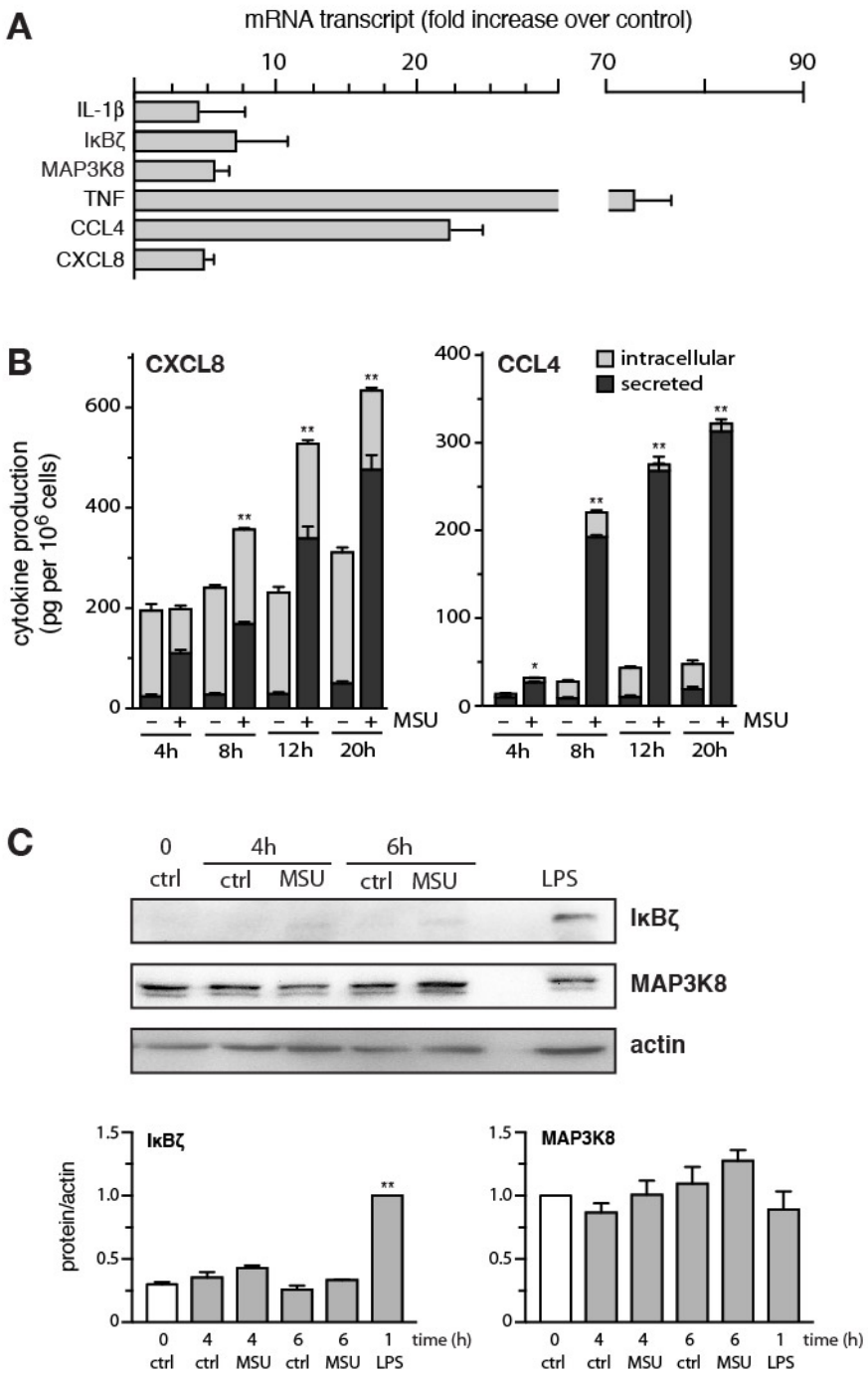


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Figure 1

A2 Figure 1. Expression of strongly induced genes, and the corresponding proteins, in MSU-activated human neutrophils.

(A) Cells were stimulated for 3 h with 1 mg/ml MSU, prior to RNA extraction, reverse transcription, and qPCR analysis. Values were normalized over RPL32 and are represented as fold increase relative to unstimulated cells. Mean ± s.e.m. from 3 independent experiments, each performed in duplicate. (B) Neutrophils were stimulated with 1 mg/ml MSU for the indicated times, prior to ELISA analysis of cell-associated chemokines and of chemokines in culture supernatants. Mean ± s.e.m. from 3 independent experiments, each performed in duplicate. *, $p < 0.05$ and **, $p < 0.01$ for total chemokine vs the respective unstimulated controls. (C) Neutrophils were cultured in the absence ("ctrl") or presence of 1 mg/ml MSU or 1 μg/ml LPS for the indicated times, prior to immunoblot analysis of cellular IκBζ, MAP3K8, and β-actin (loading control). A representative experiment is shown, along with compiled data from at least 3 independent experiments. **, $p < 0.001$ vs unstimulated control.

We next investigated whether the corresponding proteins were also upregulated in MSU-treated neutrophils. Cells were cultured for increasing lengths of time with the crystals, prior to ELISA or immunoblot analysis of the proteins of interest. As shown in Fig 1B, substantial amounts of CXCL8 and CCL4 were synthesized and secreted over time. Initially, most of the released CXCL8 came from preformed pools of the chemokine, whereas the later secretion of CXCL8 predominantly involved newly synthesized CXCL8 (Fig 1B). This is in contrast with the secretion of CCL4, which largely reflects the accumulation of newly-made chemokine (Fig. 1B). By comparison, IL-1α/β or TNFα production was either undetected or at the detection limit at 20 h (data not shown). Finally, cellular levels of MAP3K8 were not significantly affected in MSU- or LPS-activated cells (Fig 2C). Cellular expression of IκBζ was also unchanged following MSU stimulation, though LPS did induce an accumulation of the protein, as expected (Fig 2C).

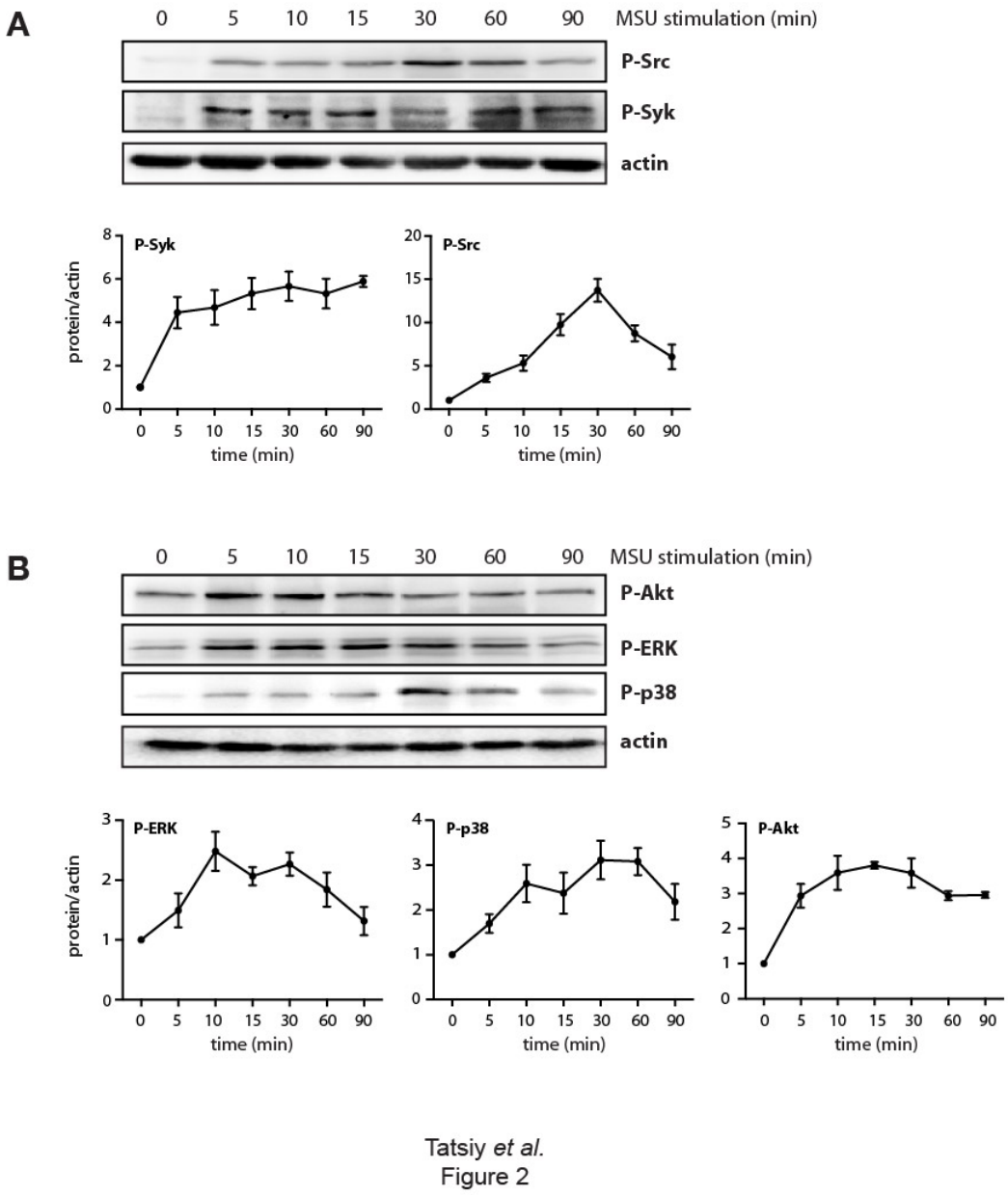

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Figure 2

A2 Figure 2. Phosphorylation of signaling intermediates in MSU-stimulated neutrophils.

Cells were stimulated with 1 mg/ml MSU for the indicated times, prior to immunoblot analysis of (A) cellular P-Src^{Y416} or P-Syk$^{Y525/526}$; (B) P-Akt^{S473}, P-ERK, or P-p38 MAPK; and β-actin (as a loading control). A representative experiment is shown in both panels, along with compiled data from at least 3 independent experiments.

Signaling cascades that are rapidly elicited by MSU. Although some signaling intermediates are known to be activated by MSU in neutrophils, the picture remains incomplete; likewise, their eventual role in neutrophil functional responses needs be elucidated. When we monitored the kinetics of various signaling pathways in MSU-treated neutrophils, we confirmed that the Src and Syk pathways are quickly activated in these cells, with phosphorylated kinases slowly returning to near-baseline levels by 90 min in the case of Src, but still elevated in the case of Syk (Fig 2A). We additionally found that MSU-stimulated neutrophils display a rapid activation of the PI3K/Akt, p38 MAPK and ERK pathways (Fig 2B), with Akt showing sustained phosphorylation at 90 min, whereas p38 MAPK and P-ERK activation appeared to be more transient. By contrast, no changes were observed in cellular IκB-α levels; similarly, no inducible phosphorylation of JNK, or of the transcriptional activators, RelA, C/EBPβ and CREB, were observed under these conditions (data not shown). Thus, a discrete set of signaling pathways seem to be mobilized by MSU in neutrophils.

We have shown previously that the p38 MAPK, MEK/ERK, and PI3K/Akt cascades are controlled by the MAP3K, TAK1, in human neutrophils exposed to various physiological stimuli (24, 25, 32, 33). We therefore verified whether this is also the case in response to MSU crystals. As shown in Fig 3A, TAK1 inhibition mostly blocked the phosphorylation of all three kinases in response to MSU. We also reported that Syk and Src family tyrosine kinases can affect at least some neutrophil responses(26, 27) and our observation that MSU rapidly activates these kinases (Fig 2A) prompted us to examine whether they may also act upstream of MAPKs and Akt. As shown in Fig 3B, Syk inhibition profoundly hindered the

phosphorylation of all three kinases, while Src inhibition only significantly affected that of p38 MAPK. Thus, both TAK1 and Syk act upstream of MAPKs and Akt, while Src family kinases contribute only to p38 MAPK activation.

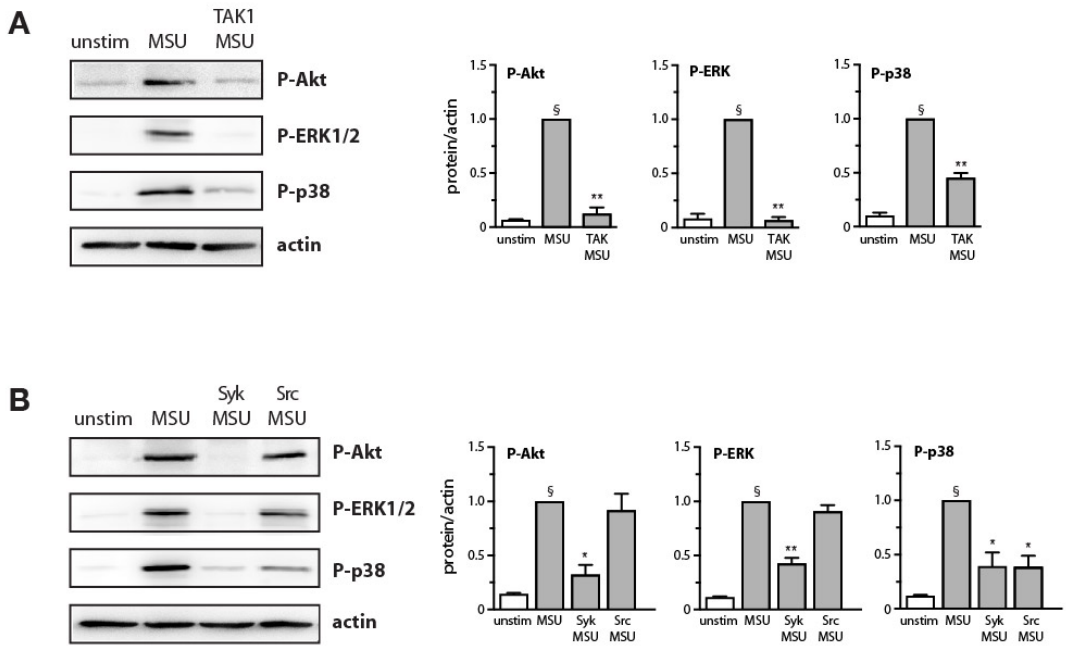

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Figure 3

A2 Figure 3. Effect of Src, Syk and TAK1 inhibition on Akt and MAP kinase activation in MSU-stimulated human neutrophils.

Cells were pretreated for 10 min in the absence or presence of (A) a TAK1 inhibitor (1 μM 5(Z)-7-oxozeaenol) or (B) a Src inhibitor (10 μM SrcI1) or a Syk inhibitor (10 μM piceatannol), prior to stimulation for 15 min with 1 mg/ml MSU or diluent control ("unstim"). Whole-cell samples were processed for immunoblot analysis of P-Akt^{S473}, P-ERK, P-p38 MAPK, or β-actin (as a loading control). Representative experiments are shown, along with compiled data from at least 3 independent experiments. §, $p<0.003$ vs unstimulated controls; *, $p < 0.05$ and **, $p < 0.01$ vs MSU alone.

Impact of signaling cascades on MSU-elicited cytokine production, and occurrence of late signaling events. We next determined which signaling pathways contribute to MSU-induced cytokine production. To this end, neutrophils were pretreated with various inhibitors, prior to stimulation for 20 h. As shown in Fig 4, inhibition of TAK1, p38 MAPK, PI3K, and Syk impaired the generation of both CXCL8 and CCL4. In contrast, inhibition of the MEK/ERK or STK pathways had no significant effect on chemokine release (Fig 4). Blocking protein synthesis with cycloheximide, or transcription with actinomycin D, confirmed that MSU-elicited chemokine secretion largely depends on their *de novo* synthesis and gene expression, respectively (Fig 4). In addition, we found that pretreating neutrophils with the NF-κB blockers, MG-132 or 15-deoxy-PGJ2, profoundly ihibited chemokine production (Fig 4).

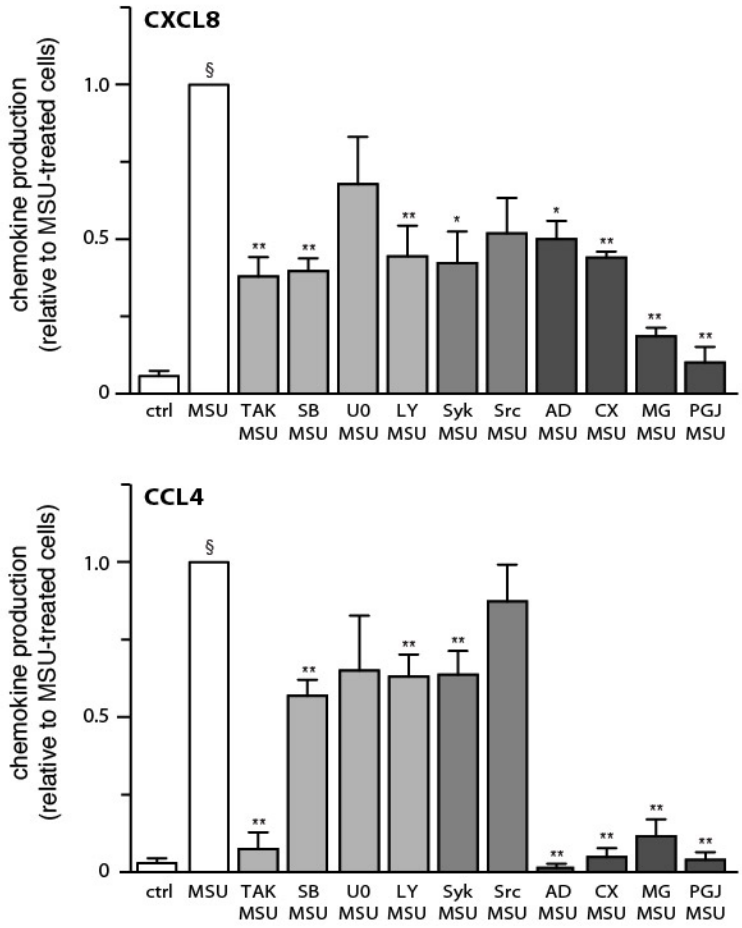

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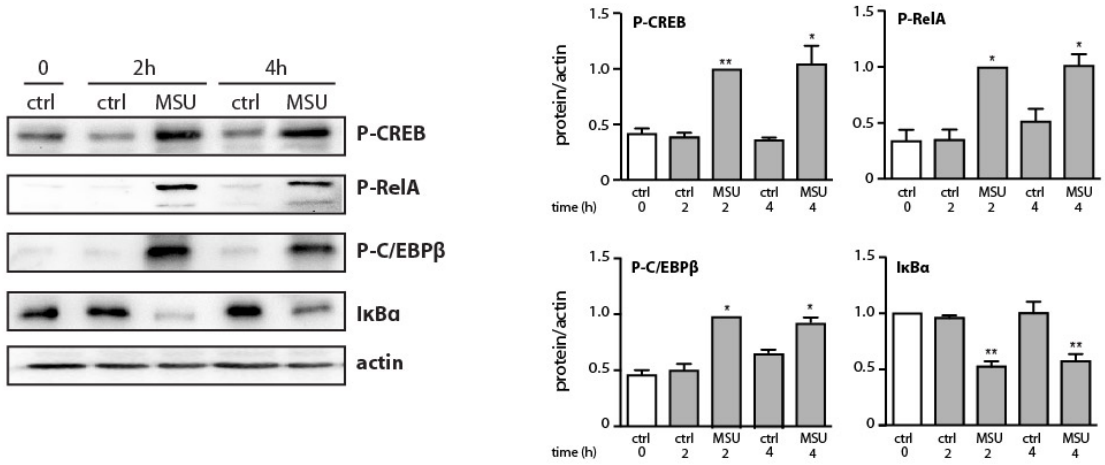
Figure 4

A2 Figure 4. Effect of various inhibitors on chemokine secretion in MSU-stimulated human neutrophils.

Cells were pretreated for 10 min in the absence or presence of inhibitors of TAK1 (1 μM 5(Z)-7-oxozeaenol), p38 MAPK (1 μM SB202190), MEK (10 μM U0126), PI3K (10 μM LY294002), Syk (10 μM piceatannol), Src family kinases (10 μM SrcI1), transcription (5 μg/ml actinomycin D, "AD"), protein synthesis (20 μg/ml cycloheximide, "CX"), or NF-κB (1 μM MG-262 or 30 μM 15-deoxy-

PGJ2). Neutrophils were then cultured in the absence ("ctrl") or presence of 1 mg/ml MSU for 20 h , prior to ELISA analysis of culture supernatants. Mean ± s.e.m. from 11 independent experiments, each performed in duplicate. Data is expressed as a ratio to MSU-stimulated cells, which amounted to 815 ± 60 pg/10^6 cells for CXCL8, and 375 ± 63 pg/10^6 cells for CCL4. §, $p<0.0001$ vs unstimulated controls; *, $p < 0.05$ and **, $p < 0.01$ vs MSU alone.

This was quite unexpected, as both inhibitors target IκB-α degradation, which we had found not to occur following MSU exposure, at least over the first 60 min of stimulation (data not shown). This notwithstanding, we also observed that few mRNA transcripts accumulate in reponse to MSU in that time frame, requiring 3 h instead to be detected in abundance (Figs S1, S2). This prompted us to investigate whether transcription factors (and associated proteins) might be activated at later time points. As shown in Fig 5A, IκB-α degradation was evident by 2 h in MSU-treated neutrophils, and IκB-α levels had still not been replenished at 4 h of stimulation. An inducible phosphorylation of transcription factors RelA, C/EBPβ, and CREB was also found to follow a similar time course (Fig 5). Thus, a belated induction of transcriptional events takes place in MSU-activated neutrophils, in keeping with the delay in gene expression.



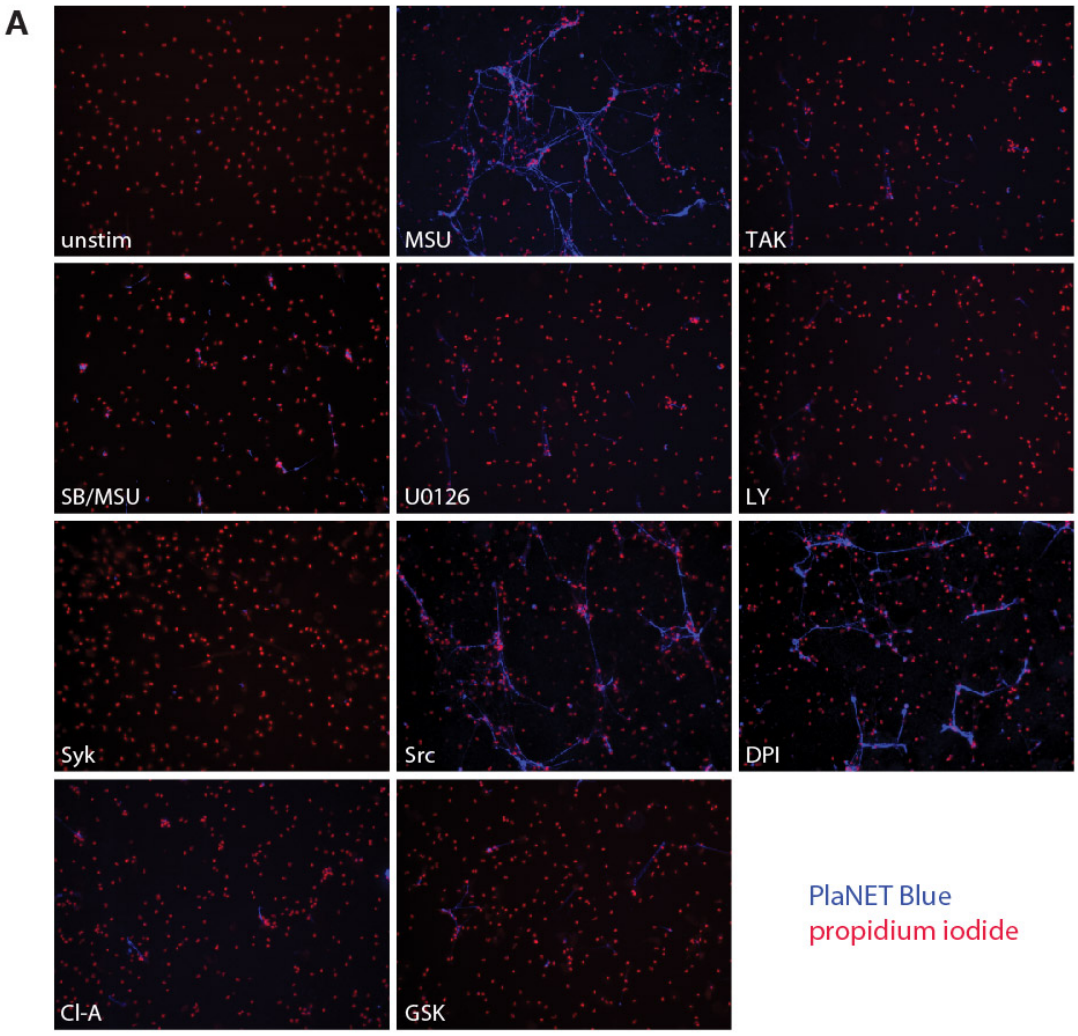
Tatsiy *et al.*
Figure 5

A2 Figure 5. Belated phosphorylation of transcription factors and associated proteins in MSU-stimulated human neutrophils.

Cells were stimulated for the indicated times in the absence ("ctrl") or presence of 1 mg/ml MSU, prior to immunoblot analysis of cellular P-CREB^{S133}, P-RelA^{S536}, P-C/EBPβ^{T235}, IκB-α, and β-actin (as a loading control). A representative experiment is shown, along with compiled data from at least 3 independent experiments. *, $p < 0.05$ and **, $p < 0.01$ vs respective unstimulated controls.

Signaling cascades involved in MSU-elicited NETosis. Besides cytokine production, another major functional response of neutrophils is their ability to form NETs (34). This phenomenon was reported to occur in response to MSU crystals (17, 18, 35, 36). To determine which MSU-elicited signaling pathways influence NETosis, neutrophils were pretreated with various inhibitors, prior to being cultured with MSU. As shown in Fig 6, inhibition of the TAK1, p38 MAPK, MEK, PI3K, and Syk pathways partially or totally prevented NET generation, whereas blocking Src family kinases had little or no effect on this response (Fig 6). Because NETosis was initially thought to depend on endogenous

ROS, and because MSU has long been known to promote the formation such molecules in neutrophils (37), we investigated whether blocking the NADPH oxidase would interfere with NET generation. As shown in Fig 6, MSU-elicited NETosis was found to be ROS-independent, but it was largely prevented by inhibition of PAD4. Collectively, the above findings shed more light on the pathways and processes controlling NETosis in MSU-stimulated neutrophils.



Tatsiy *et al.*
Figure 6

A2 Figure 6. Signaling pathways controlling NET formation induced by MSU.

Neutrophils cultured on poly-L-lysine-coated coverslips were pre-treated (15 min, 37°C) with the following inhibitors or their diluent (culture-grade DMSO): 1 μM (5Z)-7-oxozeaenol (TAK1 inhibitor); 1 μM SB202190 (p38 MAPK inhibitor); 10 μM U0126 (MEK inhibitor); 10 μM LY294002 (PI3K inhibitor); 10 μM piceatannol (Syk inhibitor); 10 μM SrcI1 (Src family kinase inhibitor); 10 μM DPI (a NADPH oxidase inhibitor); 10 μM chloraminidine ("Cl-A", a general PAD inhibitor); or 10 μM GSK484 (a PAD4 inhibitor). The cells were then further incubated for 4 h in the absence ("ctrl") or presence of 1 mg/ml MSU. NETosis was assessed using PlaNET Blue as described in *Methods*. (A) Representative fields for each experimental condition are shown at 10X magnification. (B) Quantitative representation of these experiments, expressed as NETosis index. Mean ± s.e.m. from at least 4 independent experiments. §, $p < 0.002$ vs unstimulated control; *$p < 0.05$ and **$p < 0.01$ vs. stimulus alone.

Discussion

Various aspects of the interaction between MSU crystals and inflammatory cells involved in gout pathogenesis have been studied in the last decades. Despite this, many gaps in our knowledge remain. In this study, we revisited the genomic and proteomic changes triggered by MSU in neutrophils, and the signaling pathways controlling MSU-elicited functional responses. This allowed us to uncover a new chemokine secreted in response to MSU; three trancription factors belatedly activated by the crystals; and signaling intermediates acting upstream of cytokine generation and NET formation.

Though some neutrophil genes were shown to be induced by MSU over the years, a systematic investigation of transcriptomic changes was (somewhat surprisingly) never undertaken. Herein, we found that unlike most neutrophil stimuli, which induce early gene expression within 30 min, MSU does not even modulate mRNA steady-state levels 2-fold over a 60-min stimulation. After 3 h however, the expression of numerous transcripts was up- or down-regulated. Among those whose accumulation was induced 3-fold or more were previously reported transcripts such as IL-1α/β and CXCL8 (12-14), but also others that

had never been observed before. Among the latter, some encode inflammatory mediators (*e.g.* CCL4, TNFα) or signaling machinery components (*e.g.* MAP3K8, IκBζ). Yet this still represents relatively few genes overall, especially when compared to classical neutrophil stimuli (such as LPS or TNFα) which, unlike MSU, strongly promote the expression of dozens of genes. Compounding the relative paucity of transcripts induced by MSU, is our observation that even fewer of the corresponding proteins actually accumulate. A striking example is that of TNFα, whose gene was induced some 70-fold, yet without any detectable accumulation of intracellular cytokine. This raises the intriguing possibility, that MSU fails to fully mobilize the translational machinery of neutrophils; studies are in progress to elucidate this conundrum. Whatever the case may be, our data represents the first report that CCL4 can be secreted by MSU-treated neutrophils. This finding has potentially important biological implications, insofar as MSU-activated neutrophils can not only contribute to their own recruitment into inflamed joints by generating CXCL8, but can also attract monocytes through their ability to secrete CCL4.

The signaling events triggered by MSU crystals in neutrophils have been only partially elucidated to date. It has been shown, for instance, that Src family kinases, Syk, PKCs, and PI3Ks are activated upon MSU challenge (19-22). We confirmed herein that Syk and Src are rapidly phosphorylated in response to MSU; whereas this response was sustained for of P-Syk (for at least 90 min), it was transient in the case of P-Src. Importantly, we found that p38 MAPK, ERK, and Akt were also rapidly phosphorylated in MSU-stimulated cells, and that the phospho-proteins were still detected after 90 min. In the case of p38 MAPK, our data confirm and extend recent observations by Rousseau *et al.* (38), who however only

detected weak p38 phosphorylation over a 5-min interval. By comparison, our data represents the first demonstration that ERK and Akt^{Ser473} can also be activated by MSU. Thus, the kinases activated by MSU are essentially the same as those mobilized by several physiological neutrophil agonists (23, 25, 26, 33, 39, 40). Morevoer, we found that the MSU-elicited phosphorylation of p38 MAPK, ERK, and Akt occurs downstream of TAK1 and Syk, much like it does in response to several classical neutrophil stimuli (24, 33). Thus, the undetectable synthesis of several proteins despite strongly induced corresponding genes in MSU-treated cells, cannot be attributed to a general defect in signaling. However, we observed that the extent to which Syk, Src, MAPKs and Akt are phosphorylated is lower in response to MSU crystals, compared to classical stimuli such as LPS and TNFα. This notwithstanding, we showed that the Syk, TAK1, p38 MAPK, MEK/ERK, and PI3K/Akt pathways all contribute to chemokine generation and/or NETosis. Therefore, even a relatively weaker activation of these kinases by MSU is sufficient to entail functional consequences. On final note, it has been reported that the MSU-elicited synthesis and secretion of IL-8 in monocytes is dependent on the activity of Src kinases and of ERK1/2 (41, 42), whereas we found herein that Src inhibition had little impact on CXCL8 generation in neutrophils. This indicates that among the various signaling pathways mobilized by MSU, different combinations contribute to a given response depending on the cell type.

Another novel finding reported herein is that the NF-κB, C/EBP, and CREB transcription factors are activated in response to MSU crystals in neutrophils. This agrees well with the fact that both CXCL8 and CCL4, whose transcripts and proteins are also induced by MSU, feature cognate binding sites for these transcription factors in their

proximal gene promoters, that are required for induction in human granulocytes (28, 29, 43). A singular characteristic of transcription factor activation by MSU, is that it was never detected at early time points (i.e within 15 min), as is the case with other neutrophil stimuli, such as LPS, TNFα, or IL-18 (28, 29, 31, 39). Instead, phosphorylation of RelA, C/EBPβ, and CREB1, as well as IκBα degradation, were only observed at 120 min and beyond. This belated activation mirrors the delayed induction of chemokine genes occurring in response to MSU, which was detected at 3 h. This is again in contrast with stimuli such as LPS, TNFα, or IL-18, which typically promote chemokine gene induction within 30 min or less. Thus, whereas a similar set of transcription factors can be activated by cytokines, TLR ligands, and MSU in neutrophils, the latter stimulus does so belatedly, resulting in the late induction of target genes. This is not due to a slow ingestion of the crystals, as the process takes place within 15 min (44). However, the mechanism remains elusive, which is a limitation of the current study.

Finally, MSU crystals proved to be powerful inducers of NET formation. Whereas other investigators had already reported that this response requires the PI3K, RIPK, and MLKL pathways (17, 45), we showed herein that it also involves the TAK1, p38 MAPK, MEK/ERK, and Syk pathways. With regard to the cellular processes governing NET formation, we observed that MSU-elicited NETosis is independent of ROS generation, confirming recent reports (36, 46, 47). Conversely, our finding that MSU-induced NET formation depends on PAD4, is to our knowledge a first. Thus, MSU appears to function like most other physiological neutrophil agonists (*e.g.* TNFα, GM-CSF, fMLP, PAF, C5a, CXCL8) with respect to the involvement of endogenous ROS and PAD4 (27). Overall, our

findings substantially extend our understanding of the mechanisms underlying NET generation by MSU crystals.

In summary, MSU crystals elicit a robust induction of a limited set of genes in neutrophils, including some that had not been reported to date (*e.g.* CCL4, TNFα, MAP3K8, IκBζ). However, only some of the corresponding proteins were similarly induced (*e.g.* CXCL8, CCL4). This involves several signaling pathways (*e.g.* Syk, TAK1, p38 MAPK, MEK/ERK, PI3K/Akt) and downstream effectors (transcription factors NF-κB, and possibly C/EBP and CREB as well). The same signaling pathways also participate in MSU-driven NET formation. Thus, our findings unveil several potentially important therapeutic targets for acute episodes of gouty arthritis, which feature a massive neutrophil influx. The fact that inhibitors for several of these molecular targets are already under active development (48-50) makes the translation to the patient more than a remote possibility.

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DISCUSSION

Neutrophils have been described as the "double-edged swords" of innate immunity in several reviews (Kaplan and Radic, 2012; Martínez-García *et al*., 2015; Parkos, 2016; Smith, 1994) as these cells aggressively protect or damage the host, depending on the physio-pathological conditions (Jones *et al.*, 2016; Mayadas *et al.*, 2014; Smith, 1994; Thieblemont *et al.*, 2016). It is important to underline that neutrophils are much more than the suicidal bacterial killers often depicted in immunology textbooks. The last decades of research provided new insights into the complex interactions in which neutrophils engage with elements of the surrounding tissue. Upon activation, they can secrete a variety of cytokines, chemokines, and lipid mediators attracting more neutrophils, as well as monocytes, lymphocytes, and DCs to the infected site (Prame Kumar *et al.*, 2018; Schuster *et al.*, 2013). Neutrophil products also support the maturation of monocytes, macrophages, NK cells and immature DCs. Additionally, neutrophils carrying phagocytized bacteria can function as antigen-presenting cells to DCs as they express MHC II (Vono *et al.*, 2017). This in turn leads to a T cell-driven response and activation of adaptive immunity, making neutrophils major players in a wide range of immune responses. In addition, recent data show that neutrophils are implicated in a broad variety of pathologies (type-1 and type-2 diabetes, psoriasis, Alzheimer, rheumatoid arthritis, gout, SLE, cancer, etc.). Likewise, neutrophil extracellular trap formation is an area of intense investigation in the context of neutrophil-related diseases.

Despite the increasingly acknowledged significance of neutrophil functional responses, some aspects thereof are still poorly understood. In this respect, our recent article stressed the urgent need to standardize the methods used for NET assessment and quantification (Article 1, Tatsiy and McDonald, 2018). In particular, current methods suffer from significant drawbacks. The biggest issue is the inclusion of an important (sometimes even predominant) positive signal. In the case of widely used NET quantification based on labeling NET-associated proteins (*e.g.* MPO or NE), we showed that NET digestion with Dnase I doesn't eliminate much of the signal. An important fluorescence signal remains near the cell surface, which may reflect the propensity of several neutrophil granule proteins associate with cell membranes upon their release from the cells (Owen *et al*., 1995; Pryzwansky *et al*., 1979). A significant false positive signal was also observed following Dnase I treatment for another popular spectrofluorometric method in which NETs are labeled using a DNA dye that is described as cell-impermeable by its manufacturer (*e.g.* Sytox Green). We also observed that neutrophil incubation with various Sytox Green concentrations commonly used for NET quantification (Gray *et al*., 2013; Gupta *et al*., 2014; Khan *et al*., 2017; White *et al*., 2016) resulted in rapid dye leaking into living cells. An important part of this thesis was spent on developing a reliable, specific, straightforward, standardized quantification method for NETs. Our approach is based on PlaNET reagents, which were developed as tools for NET research in the course of our investigations. These reagents now provide a viable solution to the methodological issues that have plagued NET research up to now, and have allowed us to study the impact of various signaling pathways on NET formation, which are summarized below. Despite its obvious advantages, the PlaNET-based method also has its limitations. Long-term neutrophil incubations (4h and more) results in some polymer uptake by neutrophils via unknow mechanisms. Although it

can be prevented by including phenylmethylsulfonyl fluoride (PMSF) in the culture medium, the continued presence of the serine protease inhibitor might affect other neutrophil responses in unpredictable ways. As a result, PlaNET-based quantification method cannot be used for kinetic studies, which require long-term neutrophil co-incubations.

Standardized PlaNET-based quantification allowed us to compare relative NET induction by various physiological stimuli. Thus, we showed that fMLP, PMA, TNF and GM-CSF are potent NET inducers while IL-8, C5a and PAF also induce NETs but to a lower extent. We also elucidated the signaling pathways that are involved, and found that inhibition of TAK1, p38 MAPK, or MEK pathways hindered NETosis in response to all physiological stimuli tested. We also demonstrated that these kinases seem to control early events (within the first 15 min) that influence the length and degree of branching of extruded chromatin filaments, as opposed to chromatin extrusion itself. By comparison, inhibiting of Syk or PI3K kinases nearly abolished NETosis by acting upon chromatin extrusion or upstream processes: this involved late signaling events (occurring at about 120 min of stimulation). This is a major new observation. However, the nature of the late processes affecting NETosis remains elusive. We could exclude newly made cytokines and chemokines as potential candidates, since neither cycloheximide (protein synthesis inhibitor) (Schneider-Poetsch *et al*., 2010) nor actinomycin D (transcription inhibitor) (Bensaude, 2011) were found to affect NETosis in response to any of the stimuli used. Finally, inhibiting Src tyrosine kinase, PKC kinase or JNK failed to prevent NETosis in response to physiological stimuli. Thus, for physiological stimuli such as TNFα, fMLP, or GM-CSF, signaling data that was obtained using PlaNET reagent are consistent with our previous findings, which showed that they can all signal

through the TAK1-MEK or TAK1-p38 axes in neutrophils (Ear *et al*., 2010; Sylvain-Prévost *et al*., 2015).

Due to the mounting evidence of NET involvement in various pathologies, our elucidation of the signaling pathways controlling NET formation has particular significance. A recent review by Boeltz *et al*., which summarized current areas of consensus and controversy about NETs, attracted attention to the poor understanding of NET formation signaling in response to physiological stimuli (Boeltz *et al*., 2019). This led these authors to conclude that unifying signaling mechanisms are not applicable to NETosis. However, we found otherwise. Using a systematic approach, we showed that there are at least some common pathways and mechanisms. In particular, our signaling data adds to the mounting evidence that PI3K/Akt and Syk pathways are crucial for NET formation regardless of the stiumuls used. Moreover, we showed that for several classes of physiological agonists, the TAK1, p38 MAPK and MEK/ERK pathways play an important role.

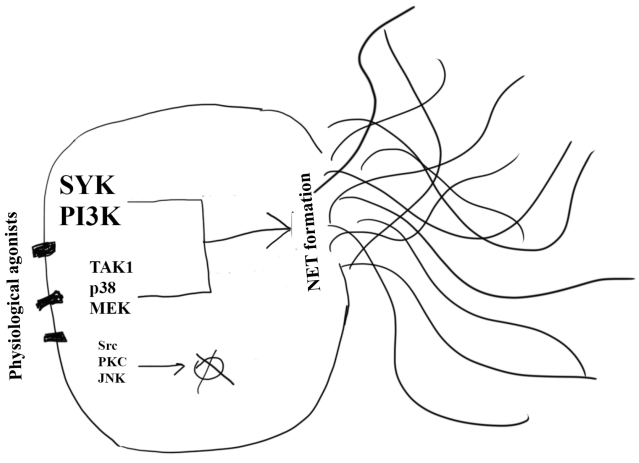

Figure 8. Common pathways of NET formation in respons to physiological agonists.

In neutrophils activated with various physiological stimuli, inhibition of TAK1, p38 MAPK, or MEK pathways hindered NETosis and effected length and degree of branching of extruded chromatin. Inhibition of Syk or PI3K kinases nearly abolished NETosis. However, inhibition of Src, PKC and JNK kinases failed to prevent NETosis in response to physiological stimuli.

Another area which we explored is the widely accepted notion that NETosis depends on endogenous ROS (Brinkmann *et al*., 2004a; Fuchs *et al*., 2007). This derives from the fact that most of the studies initially performed used PMA as a stimulus, which is a strong NADPH oxidase activator (Karlsson *et al*., 2000). Nevertheless, it was later shown that NETs can be formed in response to stimuli (GM-CSF, TNF, calcium ionophore, etc.) that are ineffective ROS inducers (Pang *et al*., 2013b; Parker *et al*., 2012b). Using PlaNET reagents, we confirmed that PMA-induced NETosis is indeed ROS-dependent. However, inhibition of NADPH oxidase didn't affect NET formation activated by various physiological stimuli (fMLP, GM-CSF, TNFα, MSU). These data are in agreement with resent observations, that NETosis can take place in ROS-independent manner (Arai *et al*., 2014; Haase *et al*., 2016; Hosseinzadeh *et al*., 2016; Kraaij *et al*., 2016; Pieterse *et al*., 2016). Thus, data obtained during the work on this thesis establishe that ROS are not essential for the NET formation in response to physiological stimuli, even though they can contribute to the process under some circumstances. Conversely, we showed that PAD4 activation is much more central mechanism for NETosis. Prior to our work, investigators had used Cl-A, which inhibits various PAD isoforms (Luo *et al*., 2006). It had been assumed that PAD4 was the isoform responsible, based on mouse studies, but no evidence had ever been generated in human neutrophils (Kolaczkowska and Kubes, 2013; Li *et al*., 2010; Martinod *et al*., 2013), which happen to express two PAD isoforms (PAD2 and PAD4) (Spengler *et al*., 2015). During the work on this thesis we used the newly available, selective PAD4 inhibitor GSK 484 (Amans *et al*., A1) to resolve the issue. We found that in human neutrophils activated by various stimuli, both GSK 484 and Cl-amidine significantly prevent NET formation to the same

extent. This demonstrated for the first time that PAD4 is the relevant PAD isoform involved in NETosis by human neutrophils.

As a result of this part of my work, we showed that there are common signaling components controlling NET formation shared across several classes of physiological NET inducers, and these pathways affect either early or late events. The nature of these early and late events has yet to be investigated. Thus, chromatin extrusion during NET formation requires 4 h and because several neutrophil products that act as NET inducers can be secreted during this time frame in stimulated cells (*e.g.* inflammatory cytokines and chemokines) (Fortin *et al*., 2009, 2011, Naegelen *et al*., 2015), we analyzed whether the inhibition of gene transcription or protein synthesis might interfere with NET formation. Nevertheless, neither process was found to be involved (Tatsiy and McDonald, 2018). While these results exclude a contribution of *de novo*-synthesized proteins to NET formation, the phenomenon could still involve pre-stored products or non-protein mediators. Future studies are needed to investigate this hypothesis.

Another part of my thesis was devoted to revisiting how MSU crystals interact with neutrophils (Article 2). Deposition of insoluble MSU in joint tissues causes gout: an acute inflammatory reaction, that is commonly associated with massive neutrophil infiltration (Martin and Harper, 2010).

We initiated our investigation by analyzing the genomic changes triggered by MSU in neutrophils. Even though gout is a prevalent disease affecting millions of people,

systematic transcriptomic analyses was surprisingly never undertaken. The DNA microarray assays that we performed demonstrated that unlike most neutrophil stimuli, which induce early gene expression within 30 min, MSU doesn't elicit significant changes in mRNA steady-state levels over a 60-min stimulation. However, after 3h of stimulation with MSU, we observed up- and down-regulation of numerous transcripts. Three-fold accumulation and higher demonstrated previously reported transcripts such as IL-1α/β and CXCL8 (Hachicha *et al*., 1995; Roberge *et al*., 1991, 1994). In addition, we observed the increased transcription of unreported inflammatory mediators (*e.g.* CCL4, TNFα) and signaling machinery components (*e.g.* MAP3K8, IκBξ). Overall however, gene induction by MSU was relatively low, compared to classical neutrophil stimuli such as LPS or TNFα. In addition, we observed that low induction of transcripts in response to MSU was accompanied by even lower accumulations of the corresponding proteins. For TNFα, whose gene was induced by almost 70-fold, no protein accumulation was detectable either extra- or intra-cellularly. This might indicate that MSU does not fully mobilize the translation machinery of neutrophils. Even though the exact mechanisms of gene induction and translation induced by MSU remain elusive, our data represent the first report that MSU-activated neutrophils secrete CCL4. This has potentially important biological relevance, as CCL4 is known to be a potent monocyte chemoattractant (Maurer and von Stebut, 2004). Thus, MSU-activated neutrophils not only contribute to their own recruitment into inflamed joints by generating high levels of CXCL8, but can also attract monocytes through their ability to secrete CCL4.

Recent studies of the signaling pathways that drive neutrophil activation by MSU crystals have led to the suggestion that Src family tyrosine kinases, Syk, PKC, and PI3Ks are

key signaling events (Popa-Nita *et al*., 2007, 2008, 2009). During the work on this thesis, we confirmed that Syk and Src are rapidly activated upon MSU stimulation; P-Syk remains sustained for at least 90 min, while the P-Src signal was transient. In addition, we demonstrated that p38 MAPK, ERK and Akt are also rapidly activated in response to MSU and that their activation remains after 90 min of neutrophil stimulation. Our data represent the first-time demonstration that ERK and Akt^{Ser473} can be activated by MSU. Although weak phosphorylation of p38 MAPK kinase was detected previously over a 5-min interval by Rousseau *et al* (2017), we demonstrated herein that activation of this kinase is much more sustained, with a peak at 30 min. In addition, we investigated upstream signaling events. Thus, we found that inhibition of TAK1 and Syk kinases strongly diminishes phosphorylation of p38 MAPK, ERK and Akt, much like it does in response to several classical neutrophil stimuli (Ear *et al*., 2010, 2017; Fortin *et al*., 2009, 2013; Sylvain-Prévost *et al*., 2015). In summary, the signaling cascades elicited by MSU are similar to those that activated in response to LPS and TNF, however the extent to which Syk, Src, P38 MAPK, ERK and Akt are phosphorylated is lower in response to MSU crystals. Although this overall weak signaling induction cannot explain why some strongly induced genes do not translate into functional proteins, it is quite obvious that even lower activity of Syk, TAK1, p38 MAPK, MEK/ERK and PI3K/Akt pathways is enough to bring about at least some functional responses (chemokine generation and NETosis). In addition, we showed that a specific Src inhibitor (SrcI1) (Bain *et al*., 2007) has no significant impact on CXCL8 and CCL4 generation by MSU activated neutrophils, whereas in monocytes MSU-elicited synthesis and secretion of CXCL8 depend on Src tyrosine kinases activity (Liu *et al*., 2001). This might indicate that various signaling pathways mobilized by MSU contribute differently to a given response depending on the cell type. However, another explanation should be taken into

consideration, as the study that demonstrated the involvement of Src tyrosine kinases in MSU-induced monocyte generation of CXCL8 used PP1 for Src tyrosine kinase inhibition. This inhibitor has been shown to nonspecifically interfere with p38 MAPK (Bain *et al*., 2007), and the latter was shown to be involved in cytokine production by monocytes in response to MSU (Chung *et al*., 2016). Thus, the results obtained might be due to nonspecific inhibition.

As we demonstrated in our research, the signal transduction cascades elicited by MSU ultimately lead to responses such as gene expression, but little is known of the transcriptional process involved. In monocytes, MSU was reported to activate the IKK/IκB/NF-κB cascade (Liu *et al*., 2001). During the work on this thesis, we showed for the first time that in human neutrophils NF-κB, C/EBP, and CREB transcription factors are activated in response to MSU crystals. This data agrees well with the fact that both CXCL8 and CCL4, whose transcripts and proteins are induced by MSU, feature binding sites for these transcription factors in their proximal gene promoters, that are required for induction in human granulocytes (Cloutier *et al*., 2009b; Ear and McDonald, 2008; Mayer *et al*., 2013). However, the activation kinetics of these TFs induced by MSU is different from the one that is typically observed in response to other neutrophil agonists. Thus LPS-, TNF-, or IL-18-induced TF activation is detected at early time points (i.e. within 15 min), while in response to MSU, phosphorylation of RelA, C/EBPβ, and CREB1, as well as IκBα degradation, were only observed at 120 min and beyond. This delayed TF activation reflects the overall delayed gene induction that is also observed in response to MSU. However, the mechanism that control rapid TF activation in

response to some stimuli (i.e. LPS, TNF) and delayed in response to others (i.e. MSU) remains to be investigated.

Finally, we demonstrated that MSU crystals are powerful NET inducers. It was shown previously that MSU-induced NETosis requires the PI3K, RIPK and MLKL pathways (Desai *et al*., 2017; Mitroulis *et al*., 2011). To study the phenomenon, we applied our standardized PlaNET-based quantification method. Results obtained revealed that TAK1, p38 MAPK, MEK/ERK and Syk pathways are important for MSU-induced NETosis. In addition, we demonstrated that MSU-activated neutrophils undergo ROS-independent NET formation, in keeping with recent reports (Chatfield *et al*., 2018; Linden *et al*., 2017; Van Avondt *et al*., 2016). Conversely, our finding that a specific PAD4 inhibitor significantly decreases NETosis in response to MSU, is the first report to our knowledge. Thus, signaling pathways that control NET formation in response to MSU appear to be similar to those utilized by other physiological stimuli employed during the work on this thesis. This lends further credence to our conclusion, that physiological stimuli use several common pathways to induce NET formation.

As a perspective for this part of my thesis, additional bioinformatic analysis of MSU-induced gene expression profile might reveal more protein candidates potentially involved in the pathogenesis of gouty inflammation. Thus, a preliminary inquiry of genes expressed by MSU-activated neutrophils using the open-source platform: reactome.org, identified gene clusters that correlate with NOD1/2, IL4, and IL13 singling pathways. Unveiled pathways were shown to play a role in the pathogenesis of various inflammatory diseases (Negroni *et al.*, 2018; Caruso *et al.*, 2014; May and Fung, 2015; Oh *et al*., 2010). However, their

intracellular signaling intermediates and role in human neutrophil functional responses is poorly understood (Ekman and Cardell, 2010; Impellizzieri *et al.*, 2019) Together, this evidence makes them prospective candidates for future MSU related research.

Understanding the molecular mechanisms of disease correspond with treatment potency. Thus, drugs currently used for the treatment of gout attacks inhibit amplification of the inflammatory response to MSU crystals. For example, colchicine, a drug with clinical efficacy in acute gout, inhibits neutrophil recruitment and activation (Ahern *et al*., 1987; Nuki, 2008; Roberge *et al*., 1994). Non-steroid anti-inflammatory agents (NSAIDs) prevent release of PGE_2 and other arachidonic acid metabolites from various cells in response to MSU crystals (Gordon *et al*., 1985; Pouliot *et al*., 1998). However, these treatments also lead to undesirable side-effects, and not all patients respond satisfactory (IQWiG, 2018). Through targeting of specific molecules and pathways involved in the initiation of neutrophil activation, as shown herein, novel treatments may be identified to prevent or treat acute gout attacks.

CONCLUSION

In present thesis, we describe a reliable and specific approach for NET quantification. This new standardized method allowed us to determine the relative potency of various physiologic NET inducers. Using newly developed standardized NET quantification method we addressed several aspects of NET formation. Thus, we showed for the first time, which PAD isoform is involved in NET formation by human neutrophils. Data obtained using specific PAD4 inhibitor confirmed that this PAD isoform is involved in NET formation in response to physiological stimuli. In addition, we determined that NADPH oxidase activation is not essential for all NET-inducing stimuli. In particular we confirmed that NET induced by physiological stimuli is largely ROS-independent. We also determined signaling pathways that are involved in NET formation. In cells activated with various physiological stimuli, inhibition of TAK1, p38 MAPK, or MEK pathways hindered NETosis by acting on early events that influence the length and degree of branching of extruded chromatin filaments, as opposed to chromatin extrusion itself. By comparison inhibiting Syk or PI3K nearly abolished NETosis; this involved late signaling events (occurring at about 120 min of stimulation), i.e. chromatin extrusion and/or upstream processes. Finally, we showed that inhibition of Src, PKC and JNK kinases failed to prevent NETosis in response to physiological stimuli. Quantification data that was obtained using PlaNET reagent substantially extends current knowledge of the signaling pathways controlling NETosis, and reveals how they affect early or late stages of the phenomenon. In view of the involvement of NETs in various pathologies, our findings also identify molecular targets that could be exploited for therapeutic interventions.

Another part of my thesis was devoted to revisiting how MSU crystals interact with neutrophils. In summary, we demonstrated that MSU crystals elicit a robust induction of a limited set of genes in neutrophils, including some that had not been reported to date (*e.g.* CCL4, TNFα, MAP3K8, IκBα). However, only several of the corresponding proteins were similarly induced (*e.g.* CXCL8, CCL4). In addition, we investigated signaling pathways that affect cytokine production and NET formation by MSU activated neutrophils. Thus, our data revealed several signaling pathways (*e.g.* Syk, TAK1, p38 MAPK, MEK/ERK, PI3K/Akt) and downstream effectors (transcription factors NF-κB, and possibly C/EBP and CREB as well) that are involved in cytokine production. In addition, we demonstrated that the same signaling pathways also participate in MSU-driven NET formation. Likewise we showed for the first time that PAD4 is involved in MSU induced NETosis. Thus, our findings unveil several potentially important therapeutic targets for acute episodes of gouty arthritis, which feature a massive neutrophil influx. The fact that inhibitors for several of these molecular targets are already under active development makes the translation (Kim and Giaccone, 2018; Li *et al*., 2018; Patnaik *et al*., 2016) to the patient more than a remote possibility.

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